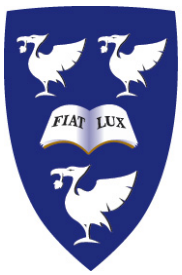

UNIVERSITY OF LIVERPOOL

Institute of Infection and Global Health
Department of Clinical Infection, Microbiology and Immunology

Imprint of Cytomegalovirus and Varicella Zoster Virus Infections on The Immune System of Healthy and Diseased Subjects

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Abstract

Primary infection with both cytomegalovirus (CMV) and varicella zoster virus (VZV) usually occurs at an early age, followed by lifelong persistent infection (latency phase). Indeed, frequent reactivation of latent viruses, particularly CMV, can be a major burden to the immune system and can play a role in configuration of the immune system.

This thesis set out with the aim of understanding the balance between different arms of the immune system and CMV and also to find out the impact of immune modulation by this virus on the sequelae of coincident coronary artery disease (CAD). Meanwhile, the purpose of the VZV study was to determine the importance of adaptive immunity in protection against VZV reactivation in children with acute lymphocytic leukaemia receiving chemotherapy.

The most obvious finding of the current CMV study is that the CMV-seropositive cohort exhibited a remarkable increase in the frequencies of $CD28^{low}$ CD4+, $CD28^{low}$ CD8+ T cells, $NKG2C^{pos}$ NK Cells and $V\delta2^{neg}$ γδ T cells compared to CMV-seronegative donors. Furthermore, it shows that the expansion of $V\delta2^{neg}$ γδ T cells in CMV carriers is age related and does not correlate with changes in the magnitude of CMV-specific CD4+ or CD8+ T cell frequencies within those individuals. Phenotypically, $V\delta2^{neg}$ γδ T cells were found to be highly differentiated effector memory cells and did not possess *ex vivo* immediate effector function. In the CAD study, the most striking observation was the differences between AMI (acute myocardial infarction) and PMI (stable post myocardial infarction) patients in term of the distributions of polyfunctional CMV-specific CD4+ T cells that were characterized by the absence of IFNγ but expression of IL-2 plus any other measured functions (i.e CD107a, MIP1β, and/or TNFα). This cell subset was significantly increased in the PMI group.

Finally, one of the more significant findings to emerge from the VZV study was the significant reduction in the absolute counts of VZV-specific IFNγ+ CD4 T cells and inversion of the VZV-specific CD4:CD8 (IFNγ producer) T cell ratio in those leukaemic children who developed clinical VZV infection. Contrasting with T cell responses, VZV-specific IgG was detected post primary infection in most patients; however, there was no clear association between the VZV-specific IgG antibody titre and VZV reactivation.

Dedication

I dedicate this work to my Father and Mother, who were always at the end of a phone with kind words of encouragement and constant support throughout this PhD. I would never have got through it without their motivations and encouragement. Also I dedicate this work to my son (Ahmed) who every day spends hours at the window waiting for me to come back from the lab!

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I would like to express my gratitude to my supervisors, Dr S Christmas and Dr B Flanagan, whose expertise, understanding, and patience, added considerably to my graduate experience. Very special thanks to my previous supervisor Dr Naeem Khan, I highly appreciate his assistance in designing and writing this thesis. I am also indebted to Dr C Gray, Dr D Howarth and MS C Broughton for their technical support and the assistance they provided at all levels during this thesis. Appreciation also goes out to Dr Q Zhang and Dr N Blake for generosity in providing reagents and for using their laboratory equipment. I must acknowledge Mrs. H Nelson for all the instances in which her assistance helped me along the way. I am also grateful to all members of the staff of Department of Clinical Infection, Microbiology and Immunology in IGH, Liverpool University for being helpful during this course.

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List of publications

Manuscript under preparation

A Alejenef, A Pacchnio, M Halawi, SE Christmas, PA Moss and N Khan (2012). *Cytomegalovirus drives Vδ2^{neg} γδ T-cell memory inflation in many healthy virus carriers with increasing age.*

Meeting abstracts

A Alejenef, S Christmas, I Hart & N Khan

The imprint of cytomegalovirus on the immune system in healthy blood donors. British society for Immunology (BSI) congress Liverpool, 2010.

A Alejenef, B Albarbar, M Caswell, B Pizer, R Keenan, S Manley, and N Khan.

Detection of Varicella-Zoster Virus-specific cellular immunity in patients with Acute Lymphoblastic Leukaemia and healthy controls. British society for Immunology (BSI) congress Liverpool, 2010.

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Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
APC	Allophycocyanin
APC	Antigen Presenting Cell
BFA	Brefeldin-A
CBC	Complete Blood Count
CCR7	CC-Chemokine Receptor 7
CD	Cluster Of Differentiation
CMV	Cytomegalovirus
CTL	Cytotoxic T Cells
DC	Dendritic Cell
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
E	Early
EBV	Epstein Barr Virus
ERGIC	Endoplasmic Reticular Golgi Intermediate Compartment
FACS	Fluorescence-Activated Cell Sorter
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
FSC	Forward Scatter
HFFF	Human Fetal Foreskin Fibroblasts
HIV	Human Immunodeficiency Virus
HLA	Human Leucocyte Antigen
HSV-1	Herpes Simplex Virus 1
HSV-2	Herpes Simplex Virus 2
HZ	Herpes Zoster
IE	Immediate Early
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRP	Immune Risk Phenotype
kB	Kilo Base
KIR	Killer-Cell Immunoglobulin-Like Receptors
L	Late
LCMV	Lymphocytic Choriomeningitis Virus
LFA-1	Lymphocyte Function-Associated Antigen 1
LN	Lymph Node
mCMV	Murine Cytomegalovirus
MFI	Mean Fluorescence Intensity
MHC I	Major-Histocompatibility-Complex Class I
MHC II	Major-Histocompatibility-Complex Class II
MIP1β	Macrophage Inflammatory Protein -1 Beta
mRNA	Messenger RNA
NK cell	Natural Killer Cell

ORF	Open Reading Frame
PAMPs	Pathogen Associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PD-1	Programmed Cell Death Protein 1
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein
pMHC	Petide MHC Complex
PRRs	Pattern Recognition Receptors
RIG-I	Retinoic Acid-Inducible Gene I
RLRs	RIG-I Like Receptors
RNA	Ribonucleic Acid
SLC	Secondary Lymphoid Tissue Chemokine
SSC	Side Scatter
TCE	T Cell Expansion
T_{CM}	Central Memory T Cell
TCR	T Cell Receptors
T_{EM}	Effector Memory T Cell
T_{EMRA}	Effector Memory CD45RA Expressing T Cell
T_H	T Helper
TLRs	Toll Like Receptors
T_N	Naive T Cell
TNF	Tumour Necrosis Factor
TRAIL	TNF-Related Apoptosis-Inducing Ligand
T_{reg} cell	Regulatory T Cell
VZV	Varicella Zoster Virus
α	Alpha
β	Beta
β_2m	Beta$_2$-Microglobulin
γ	Gamma
δ	Delta
ε	Epsilon
ζ	Zeta
μ	Micro

1 General Introduction

In this introduction chapter, the basic virology of herpesviruses (CMV and VZV) as well as immune responses to both viruses will be discussed. However, there will be more focus on cell-mediated immunity particularly that related to CMV infection as most of work in this thesis is related to this virus.

1.1 Virology

Herpesviruses (also called Herpesviridae) are a large group of DNA viruses. According to their biological function and sequence similarity, they can be classified into three major subfamilies, namely; Alpha (α), Beta (β) and Gamma (γ) (Arvin, 2001, Pellett and Roizman, 2007, Davison *et al.*, 2009). In humans, eight different herpesviruses were found to cause diseases, see Table 1-1. Each one of these viruses preferentially infects a specific type of cell whose defence mechanisms the virus can circumvent (permissive cells). Virus replication in the infected cells causes cell death and lysis; therefore, herpes infection is described as lytic infection. Furthermore, almost all herpesviruses can establish dormant infection where no viral replication in blood can be detectable (latency) (Pellett and Roizman, 2007). See Table 1-1.

Table 1-1: Summary of characteristics of herpesviruses that infect humans

Name	Subfamily	Cell types infected		Pathophysiology
		Lytic infection	Latent infection	
HHV1 (HSV-1)	α	Epithelial cells	Neurons	Orofacial infections, encephalitis
HHV2 (HSV-2)	α	Epithelial cells	Neurons	Genital and neonatal infections
HHV3 (VZV)	α	Epithelial cells	Neurons	Chickenpox, shingles
HHV4 (EBV)	γ	B cells, epithelial cells	B cells	Infectious mononucleosis, lymphoma, carcinoma
HHV5 (CMV)	β	Macrophages, lymphocytes, epithelial cells	Macrophages, lymphocytes, epithelial cells	Congenital infection, retinitis, hepatitis
HHV6	β	$CD4^+$ T cells	Monocytes, macrophages	Exanthem subitum
HHV7	β	T cells	T cells	Exanthem subitum
HHV8	γ	Lymphocytes	Lymphocytes	Kaposi's sarcoma

CMV, cytomegalovirus; EBV, Epstein–Barr virus; HHV, human herpesvirus; HSV, herpes simplex virus; VZV, varicella-zoster virus.

This thesis focuses on investigating the immunology of two common herpesviruses, cytomegalovirus (CMV) and Varicella zoster virus (VZV). The reasons behind this were an increasing appreciation of their role in configuration of the immune system during health and their association with morbidity and mortality during disease (Khan, 2007, Gluckman, 2009, Sengupta and Breuer, 2009). In addition, both of these two viruses have high prevalence in the UK, for instance, the CMV prevalence was found to increase with age from approximately 15% in those aged 1-4 years to approximately 80% in those aged over 64 years (Vyse *et al.*, 2009). In contrast, by the age of 5 years, approximately 77% of children will be VZV seropositive; meanwhile 95% of adults can be seropositive (Manikkavasagan *et al.*, 2010).

1.1.1 Herpesvirus structure

In order to understand the immune response to CMV and VZV herpesviruses, it is essential to understand both the viral particle structure (virion) as well as the replication cycle of the virus. Starting with the virion structure, as can be seen in Figure 1.1, the virions of both CMV and VZV have a typical herpesvirus structure. The outside envelope consists of a lipid bilayer derived from the host cell endoplasmic reticular (ER)-Golgi intermediate compartment (ERGIC) with virus-encoded glycoprotein. In CMV these glycoproteins are classified into three sub groups: glycoprotein class I, glycoprotein class II and glycoprotein class III (gB, gH:gL and gM:gN respectively) (Yurochko *et al.*, 1997, Vanarsdall *et al.*, 2008). In VZV, there are gB, gC, gE, gH, gI, gK and gL as well as the putative glycoproteins gM and gN (Arvin *et al.*, 1986, Oxman, 2009). As will be discussed in the replication cycle (1.1.2), the main role of these proteins is helping attachment of viruses to host cell membranes.

Immediately inside the envelope, there is the tegument, which is also known as the matrix. It contains most of the virion proteins as well as viral and cellular RNA. In CMV at least 27 relatively abundant virus encoded proteins are present in the tegument, the most abundant protein is pp65 (product of the UL83 gene) followed by pp71 (product of the UL82 gene) and pp150 (product of the UL32 gene), respectively (Tomtishen, 2012). Meanwhile in VZV, it contains proteins encoded by open reading frames (ORF) 10, 47, 62 and 63 (Besser *et al.*, 2004). In addition to the structural role of tegument proteins, they also play an essential role during the viral replication cycle. For instance, it is believed that tegument proteins perform two main roles during the replication cycle; firstly, they modulate the host cell response to virus invasion by blocking intrinsic host defences and therefore optimizing the intracellular environment for virus replication. The second role is initiation of virion assembly and disassembly during entry stage as well as during the final envelopment and egress from the cytoplasm (Ostrove, 1990, Liesegang, 1992).

Both CMV and VZV genomes consist of linear, double-stranded DNA. In CMV, the genome consists of approximately 235kBp and encodes more than 170 genes. Meanwhile in VZV, it is only 124kBp and encodes around 70 unique genes (ORF); most of them are still undefined in VZV. DNA is separated from the matrix by an icosahedral nucleo-capsid (Knipe and Howley, 2007)

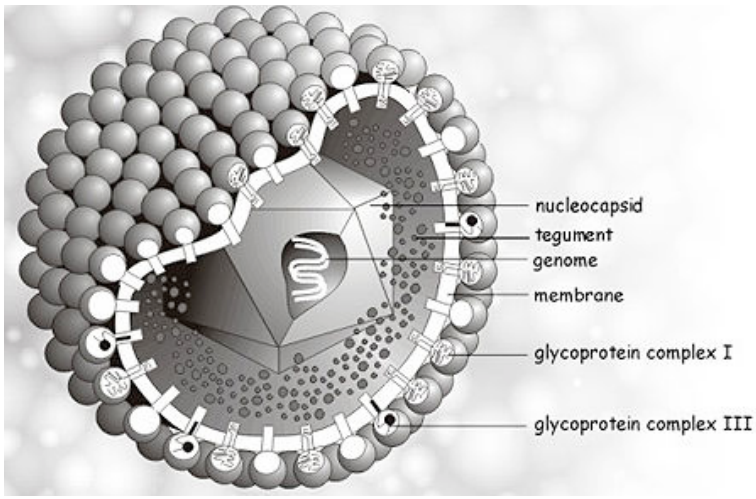

Figure 1.1: Schematic of herpesvirus particle

This figure shows a schematic representation of the herpesvirus particle. A linear double stranded DNA genome is covered by capsid proteins, which are surrounded by a matrix of protein (tegument). There are several glycoproteins embedded in the outer bilayer envelope membrane (Image obtained from (http://www.virology.net/big_virology/bvdnaherpes.html website) courtesy of Dr. Marko Reschke, Marburg, Germany).

1.1.2 Replication cycle

The replication cycle of CMV and VZV follows a sequential pattern of gene expression common to all herpesviruses. For instance, within the first 2 hours of infection, transcripts classed as immediate-early (IE) genes are made. The IE products, IE-1 and IE-2, are transcription factors that drive the expression of early (E) genes and, much later, the expression of late (L) genes. Therefore, the replication cycle can be divided into three different phases; Figure 1.2 summarises these steps.

I. Virus adsorption and entry, uncoating, transportation of the capsid to the nucleus and release of the viral DNA into it,

II. Viral gene transcription and translation as well as synthesis of viral DNA and

III. Assembly of new virions, enveloping and egress.

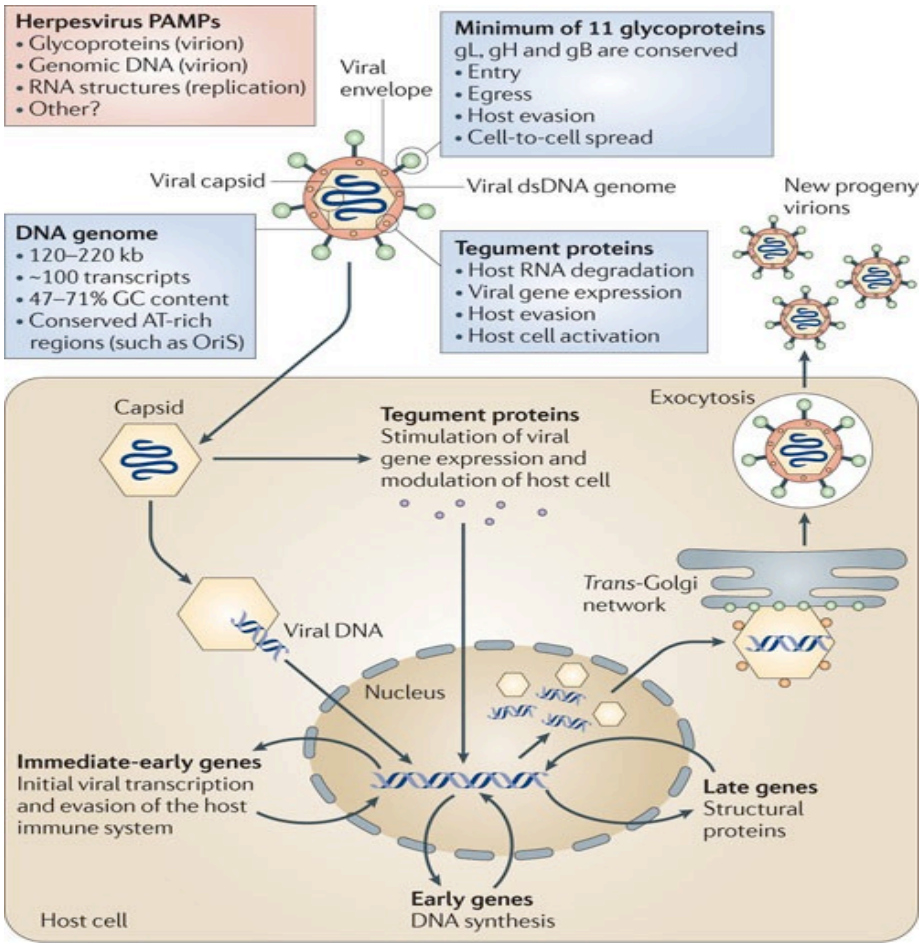

Figure 1.2: Replication cycle of herpesviruses.

This Figure illustrates the replication cycle of herpesviruses. Specific glycoproteins in the viral envelope help in attachment to the host cell membrane. Penetration is achieved when the viral envelope fuses with the cell membrane releasing the nucleo-capsid directly into the cytoplasm. To ensure transport and docking of the virion at nuclear pores, after uncoating some encoded viral glycoproteins and tegument proteins modulate the cell environment and prevent internal cell defence against the virus. In the nucleus, viral DNA is replicated into many copies. The viral DNA is then transcribed into the late mRNAs, which exit to the cytoplasm for translation into the late (nucleo-capsid and envelope) proteins. At the same time, the capsid proteins encapsidate the newly replicated genomes. The envelope proteins are imbedded in the nuclear membrane. The nucleo-capsid is enveloped by budding through the nuclear membrane, as well as endoplasmic reticulum and Golgi apparatus. Finally, the mature viruses are released from the cell into intercellular spaces. Figure adapted from (Paludan *et al.*, 2011)

1.1.3 Transmission and Epidemiology

1.1.3.1 Cytomegalovirus (CMV)

In the context of CMV infection, it is important to emphasize that the term 'CMV latency' here does not mean that the virus is completely in an inactive mode. In fact, there is a continuous but low level of virus replication that is usually undetectable but cannot escape the immune system surveillance. Supportive evidence of this comes from the possibility of isolating virus particles from a variety of body fluids. For instance, in addition to semen and vaginal secretions, CMV was detected in 61% of saliva samples, 10% of uterine secretions and 37% of urine samples (Gautheret-Dejean *et al.*, 1997). Therefore CMV infection can be transmitted by several means including blood transfusion, transplantation, nursing and sexual contact. Another possible route of CMV transmission is the vertical transmission from mother to child, particularly during delivery. Although CMV is found throughout all geographic locations and socioeconomic groups, incidence is higher in developing countries and among low socio-economic classes. Furthermore, the prevalence of infection is found to be age-dependent, where the proportion of seropositivity increases with advancing in age (Gautheret-Dejean *et al.*, 1997, Staras *et al.*, 2006).

1.1.3.2 Varicella zoster virus (VZV)

Compared to CMV, primary VZV infection is usually acquired during childhood. This might be explained as primary infection is highly contagious from child to child by direct contact, breathing aerosols from vesicular fluid of skin lesions, or infected respiratory tract secretions that are aerosolized by the transmission of virus. In contrast the incidence of reactivation (herpes zoster) increases with age, for instance, nearly 40%

to 50% of the one million new cases that appear annually are individuals over 50 years of age who develop zoster (Gauthier *et al.*, 2009). Conversely to primary infection, secondary infection is also transmitted from person to person by direct contact, because of its respiratory transmission. Nevertheless the risk of transmission of zoster infection is less than that of chickenpox infection.

1.1.4 Clinical presentation of CMV and VZV infection

1.1.4.1 CMV clinical manifestation

In healthy subjects, infection with CMV is typically self-limiting with very mild flu-like symptoms and usually passes without complication. However, in immunocompromised subjects, including patients with congenital and acquired immunodeficiency, CMV can cause a wide range of clinical syndromes. This might include retinitis (which may cause blindness), pneumonitis, cholangitis, hepatitis, gastrointestinal ulcerations, colitis, abdominal pain, encephalitis, fever and weight loss (Knipe and Howley, 2007). Although the majority of CMV infections are attributed to reactivation of latent virus, the acquisition of new infection is of equivalent importance. For instance 30% of seronegative recipients receiving kidneys from seropositive donors become seropositive, and mortality was as high as 80-90% in such groups before the development of anti-viral drugs (Vancikova and Dvorak, 2001).

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1.1.4.2 VZV clinical manifestation

Varicella-zoster virus (VZV) infection is usually a symptomatic disease. It has two clinically different forms of the disease; primary infection with VZV results in varicella (chickenpox), characterized by vesicular lesions in different stages of development on the face, trunk, and extremities. Meanwhile, herpes zoster (HZ), which also is known as shingles, results from reactivation of endogenous latent VZV infection within the sensory ganglia. Clinically HZ is characterized by a painful unilateral vesicular eruption, which usually occurs in a restricted dermatome distribution, see Figure 1.3.

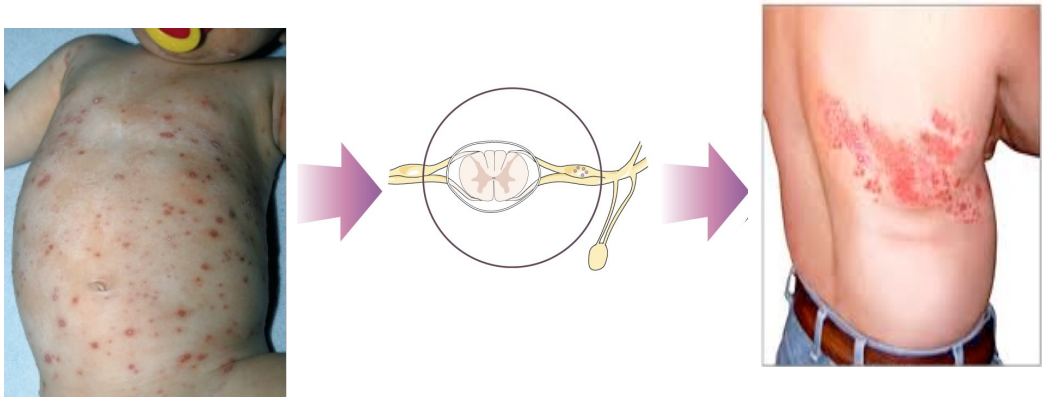


Figure 1.3: Primary VZV infection (chickenpox) and VZV reactivation (herpes zoster)

Represents the two different diseases associated with VZV infection. Primary infection VZV in children on the left hand side and secondary VZV herpes zoster that emerges in elderly people on the right hand side. The figure was modified from, Lancet 2006 368: 1365–76, and Arvin AM. Varicella-zoster virus. In: Knipe DM, Howley PM, eds. Fields Virology. Vol 2. 4th ed.

1.1.5 Hazards of congenital and neonatal infection by CMV or VZV

CMV is the most common infectious cause of congenital defects and it is important to appreciate the hazards of this virus. Congenital CMV infection takes places when pregnant women experience a primary infection or to a lesser extent a reactivation during pregnancy (Grant *et al.*, 1981). It has been reported that the incidence of primary CMV infection in pregnant women in the UK varies from 1% to 3%, whereas more than 40% of neonates from such women will suffer no symptoms at birth. Meanwhile less than 10% are symptomatic, which may range from mild hearing loss to severe mental retardation and disability (Peckham *et al.*, 1983, Stagno and Whitley, 1985b, Stagno and Whitley, 1985a, Freij and Sever, 1988, Pass *et al.*, 2006). Somewhat surprisingly, the incidence of congenital CMV is found to be slightly higher in developed countries compared to developing countries. This can be explained by the fact that in developed societies the sero-prevalence of CMV is much lower than that in developing ones, where more women of child-bearing age are already CMV-seropositive (Malm and Engman, 2007). In other words, most mothers in developing countries already have high titres of anti–CMV antibodies that can protect their foetuses from being harmed by the virus. In contrast, in developed countries if mothers catch the virus for the first time during pregnancy they will not have enough time to develop a humoral immune response to CMV. Thus, the virus will be transmitted at a higher rate than development of the protective antibodies, and greater severity of foetal tissue damage may occur. Nevertheless, the presence of antibodies in the urine of 1% of healthy neonates reveals that, as in adults, the disease often passes unnoticed (Peckham *et al.*, 1983, Pass *et al.*, 2006).

Since most women would be naturally immunized against VZV by childbearing age, then the risk of congenital varicella is quite low and the overall risk of infection is quite small compared to herpesviruses. However, most cases of congenital varicella syndrome occur in infants whose mothers were infected between 8 and 20 weeks of gestation. The risk seems to be approximately 2% if the infection occurs before 20 weeks of gestation and less than 1% if it occurs before 13 weeks (Enders *et al.*, 1994, Pastuszak *et al.*, 1994).

1.2 Immunology

Although both innate and adaptive immune responses are involved in controlling CMV and VZV infection, in this review more focus is given to cellular elements of the adaptive immune system as it is more related to the work in this thesis. In the following sections, the role of the innate immune system in recognition and presentation of viral antigen to cells of the adaptive immune system will be discussed in more detail.

1.2.1 Recognition of herpesviruses by the innate immune system

Briefly, the innate immune system is activated when the infection is sensed by pattern recognition receptors (PRRs), through detection of specific components of the virion known as pathogen associated molecular patterns (PAMPs). Several PRRs are involved in recognition of CMV and VZV infection, however among PRRs the most studied are Toll Like Receptors (TLRs) (Takeuchi and Akira, 2010). Of particular importance is TLR2 that is integrated in the plasma membrane and can sense gB or gH of CMV and virion components in VZV (Wang *et al.*, 2005, Boehme *et al.*, 2006). In addition,

herpesvirus genomic DNA can be recognised by cytoplasmic TLRs such as TLR9 (Tabeta *et al.*, 2004), as well as by endosomal TLRs that can sense nucleic acids.

Besides TLRs, there are other PRRs that can recognize RNA produced during herpesviral replication, which involve RIG-I like receptors (RLRs) and retinoic acid-inducible gene I (RIG-I) (Paludan *et al.*, 2011). Upon sensation of herpes infection, almost all PRRs induce intracellular signalling pathways that lead to expression of pro-inflammatory cytokines such as type I IFN (IFNα and IFNβ). These cytokines have an important role in blocking of local spread of virus through inducing a state of viral resistance in adjacent non-infected cells. Furthermore, as will be seen later, these cytokine are important for the activation of the adaptive immune system.

1.2.1.1 Role of natural killer (NK) cells in immunity to CMV and VZV

In the innate antiviral immune response, type I IFN and natural killer (NK) cells have key roles in protection against CMV and VZV infections. For instance, observations from the murine model of CMV infection and to a lesser extent from human clinical reports demonstrate the importance of NK cells in early control of CMV infection. An example of this is certain strains of mice that are resistant to mCMV (Murine Cytomegalovirus) that become susceptible to infection upon interference with NK cells, either by NK cell depletion or through genetic modification of NK cell function (Brown *et al.*, 2001). This also seems to be true in humans where several reports have shown that recurrent CMV infection can occurs in patients with NK cell deficiency, this reviewed in great details by Orange (Orange, 2002). Similarly, an association between severe VZV infections and NK cell deficiency has been reported (Notarangelo and Mazzolari, 2006). In addition, NK cells were found to be able to lyse VZV-infected targets (Ihara *et al.*,

1991) and blocking of IFNα, for which NK cells can be one of its sources, was associated with spread of VZV in the skin (Ku *et al.*, 2004).

The mechanisms that are used by NK cells to defend the body against herpesviruses are still not fully understood. However, the principle of self-recognition and altered self-state are thought to be involved. It is known that NK cells possess two types of surface receptors: activating receptors such as CD94/NKG2C and inhibitory receptors such as CD94/NKG2A and Killer-cell Immunoglobulin-like Receptors (KIR). The activity of NK cells is determined by the balance between the activities of these receptors. In normal circumstances, when host cells are healthy, the inhibitory receptors recognize MHC class-I (MHC-I) alleles, and send an inhibitory signal, and therefore the NK cells are prevented from being activated (Farrell and Davis-Poynter, 1998, Tortorella *et al.*, 2000, Khan, 2007). Nevertheless, as a strategy of CD8 T cell evasion many viruses including the herpesviruses have the ability to down regulation of MHC-I. However, this loss of MHC-I would deprive these cells of the inhibitory effect of MHC-I/NK inhibitory receptors and render these cells vulnerable to NK cell mediated lysis (Wilkinson *et al.*, 2008). How the herpesviruses avoid detection by NK cell will be discussed late in section 1.2.5.

1.2.2 Recognition of herpesviruses by the adaptive immune system

Collectively, B cells (humoral immunity) and T cells (cell-mediated immunity) are called ‘the adaptive immune system’. The hallmarks of adaptive immunity are antigen specificity and memory, which provide unique mechanisms for better protection. Indeed, both humoral immunity and cell-mediated immunity are required for effective response against herpesvirus infections. However, since intracellular infection is obligatory for

herpesvirus infection, cell-mediated immunity seems to be more important for clearance of established viral infection. Therefore, in this review more attention will be paid to cell-mediated immunity.

1.2.2.1 Cell mediated immunity in herpesvirus infection.

Unlike antibody, which can recognize complete viral antigens, conventional αβ T cells (CD4 and CD8 T cells) cannot recognize intact viral proteins. Instead, their T cell receptors (TCR) sense only special epitopes of the short viral peptides that are presented by antigen presenting cells (APCs) in association with major histocompatibility complex (MHC) molecules. In addition, involvement of non-conventional T cells such as γδ T cells in responses against herpesviruses, particularly CMV, has become evident. For appropriate understanding of cell-mediated immunity, the classification of T cells according to the TCR structure will be discussed in the next section.

1.2.2.2 T cell receptor structure (TCR)

Generally the TCR consists of two heterodimer chains that are disulphide-linked and anchored to the cell membrane (Figure 1.4). Each chain is composed of a Variable (V) region and a Constant (C) region. The Constant region anchors the TCR to the cell membrane, including a short cytoplasmic tail, while the extracellular Variable region binds to the antigen:MHC complex. As its name indicates, the TCR of αβ T cells is composed of alpha (α) and beta (β) chains; meanwhile, in a minority of cells, the receptor is formed by γ and δ chains, and thus referred as γδ T cells.

The propagation of signal from activated TCR into the intracellular compartment is facilitated through accessory molecules. These include; two pair of Zeta chains (ζ) and CD3, which possesses three distinct chains (γ, δ, and ε). Together The CD3- and ζ-

chains with either αβ-TCR or γδ-TCR, form what is known as the T cell receptor complex. Furthermore, according to expression of CD4 or CD8 co-receptor the αβ T cell is classified into CD4+ and CD8+ T cells respectively.

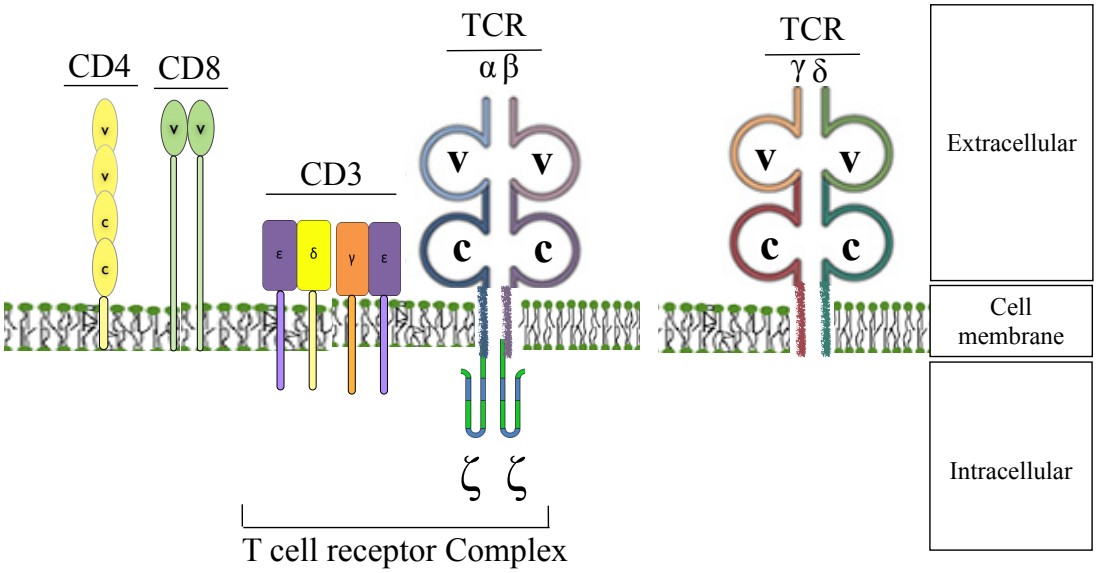


Figure 1.4: T cell receptor complex structure

This illustrates the T cell receptor complex structure. TCR is composed of two protein chains (α/β or γ/δ) and each chain is composed of a Variable (V) region and a Constant (C) region. The TCR-complex includes αβ-TCR or γδ-TCR plus signal transduction molecules. These accessory molecules consist of two pairs of Zeta chains (ζ) and CD3, which possesses three distinct chains (γ, δ, and ε). In addition, αβ T cells express either CD4 or CD8.

1.2.2.3 Comparison between αβ and γδ T cells

Contrasting with αβ T cells, γδ T cells are predominantly CD4− CD8− double negative (Raulet, 1989, Chien and Bonneville, 2006). Table 1-2 below shows more comparisons between αβ and γδ T cells. In the peripheral circulation, γδ T cells are a small subset of T cells, approximately 2-10% of total T cells. However, in gut mucosa γδ T cells form a higher proportion of the intra-epithelial lymphocyte population, where they reach 10-50% of the T cell pool (Raulet, 1989, Deusch *et al.*, 1991, Hayday, 2000). Notably, there is also another sub-population of γδ T cells that reside in the epidermal compartment of the skin, which are known as Dendritic Epidermal γδ T cells (DETC), better characterised in mice.

Table 1-2: Comparison between αβ and γδ T cells

Feature	αβ T cells	γδ T cells
Phenotype	CD3+ CD4+/- CD4 CD25+ CD8+/-	CD3+ CD4-CD8- (double negative)
Distribution	90 -95% of peripheral blood lymphocytes	2-10% of peripheral blood lymphocytes, however 10-50% of intraepithelial lymphocytes. Also in epidermis (DETC).
Antigen recognition	Recognize peptide antigens that are processed and presented via the MHC-I or II of antigen presenting cells (APC)	Can recognize lipid & phosphorylated antigens directly and independent of MHC
Function	CD4 T cell mainly helper CD8 T cells mainly cytotoxic CD4 CD25+ regulatory	Seems to have important role in defence against bacterial, parasites and viral infection. As well as regulatory function
Repertoire diversity according to TCR V (D) J gene segments recombination	Vast repertoire due to high number of genes that are available for recombination; **Vα= 41** / **Vβ= 30** **Jα= 61** / **Dβ= 2** **Jβ= 14**	Relatively limited repertoire with less genes available for recombination; **Vδ= 3** / **Vγ= 5** **Dδ= 3** / **Jγ= 3** **Jδ= 4**

Table modified from (Girardi, 2006)

Our understanding of γδ T cell biology spans definitions of both innate and adaptive immune responses. For instance, after acute response to infection, γδ T cells are found to develop a memory phenotype resembling that of B and T lymphocytes (Hoft *et al.*, 1998). Besides, they can rearrange TCR genes to produce junctional diversity, which again is a typical characteristic of cells of the adaptive immune system. On the other hand, the recognition of common microbial molecules by using restricted T cell receptor (TCR) as pattern recognition receptor is, indeed, one of the common features of innate immune system cells.

Based on which TCR gene segment is expressed, γδ T cells can be classified into several subclasses. However, for simplicity in this thesis they will be divide into two main subclasses; Vδ2 positive γδ T cells ($V\delta2^{pos}$ γδ T cell) and Vδ2 negative γδ T cells ($V\delta2^{neg}$ γδ T cell). One of the most common $V\delta2^{pos}$ subclasses in peripheral blood is Vγ9/Vδ2 T cells, which represents approximately 70% of γδ T cells in peripheral blood. These cells can proliferate significantly in many acute infections. This proliferation is particularly clear in the early stages of infection, when the proportion of Vγ9/Vδ2 T cells even may exceed that of other lymphocytes such as in tuberculosis, listeriosis and malaria infections. Furthermore, they are able to kill tumour cells such as myeloma and lymphoma cell lines (Ferrarini *et al.*, 2002).

In contrast, $V\delta2^{neg}$ γδ T cells are referred to as γδ T cells that do not express a Vδ2 chain. Instead, they usually express a Vδ1 chain and to a lesser extent Vδ3 with any Vγ chain combination, see Figure 1.5. These cells are mainly located in peripheral tissues, particularly in spleen and the intra-epithelial compartment with very low percentages in

peripheral blood. Furthermore, Vδ2^{neg} γδ T cells were found to express spontaneously high levels of granzyme-B and perforin, which could explain their ability to kill CMV-infected cells and hence the ability to inhibit CMV propagation.

Though the particular mechanism of how γδ T cells can respond against various pathogens is not fully understood, a recognition of small non-peptide common microbial compounds was proposed (Bonneville and Fournié, 2005). For instance, Vγ9/Vδ2 T cells can recognize (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), which is formed during biosynthesis of isopentenyl pyrophosphate (IPP) by the non-mevalonate pathway. The IPP compound is vital to most pathogenic microbes including *Mycobacterium tuberculosis* and malaria, but not found in humans (Morita *et al.*, 2007). Therefore it is not surprising to see no response of Vγ9/Vδ2 T cells towards those microbes that synthesise IPP depending on the classic mevalonate pathway, where no HMB-PP is formed. Typical examples of microbes that do not activate Vγ9/Vδ2 T cells are streptococcus, staphylococcus and borrelia.

Several ligands and receptors have been proposed for Vδ2^{neg} γδ T cells activation. These include stress-induced antigens expressed on epithelial tumour cells, some leukaemias, and lymphomas, examples of these are UL16-binding proteins (Catellani *et al.*, 2007) and MHC class I-related chains (MICA and MICB) (Zhao *et al.*, 2006). Both of them can serve as ligands for an activating Natural killer-G2D receptor (NKG2D). Moreover, very recently, Knight and colleagues have showed Vδ1^{pos} γδ T cell cytotoxicity against myeloma cells was mediated in part through the T cell receptor (TCR) in addition to involvement of NKG2D, DNAX accessory molecule-1 (DNAM-1), intracellular cell adhesion molecule (ICAM)-1, CD3 and CD2 receptors (Knight *et al.*, 2012).

Furthermore, Vδ1^{pos} γδ T cells can directly mediate killing of leukaemia cell lines and chronic lymphocytic leukaemia patient neoplastic cells through natural cytotoxicity receptors (NCRs) including NKp30, NKp44 and NKp46 (Correia *et al.*, 2011)

Although, the role of Vδ2^{neg} γδ T cells, in CMV infection has become evident, the exact molecules responsible for activation of Vδ2^{neg} γδ T cells have not been precisely identified. However, the mechanism of recognition of CMV-infected cells is thought to be mediated by the γδ TCR, as the effect could be blocked using anti-TCR antibodies. Furthermore, similar to other sub-classes of γδ T cells, Vδ2^{neg} γδ T cells do not require MHC-I presentation; nevertheless direct contact may be needed for killing activity.

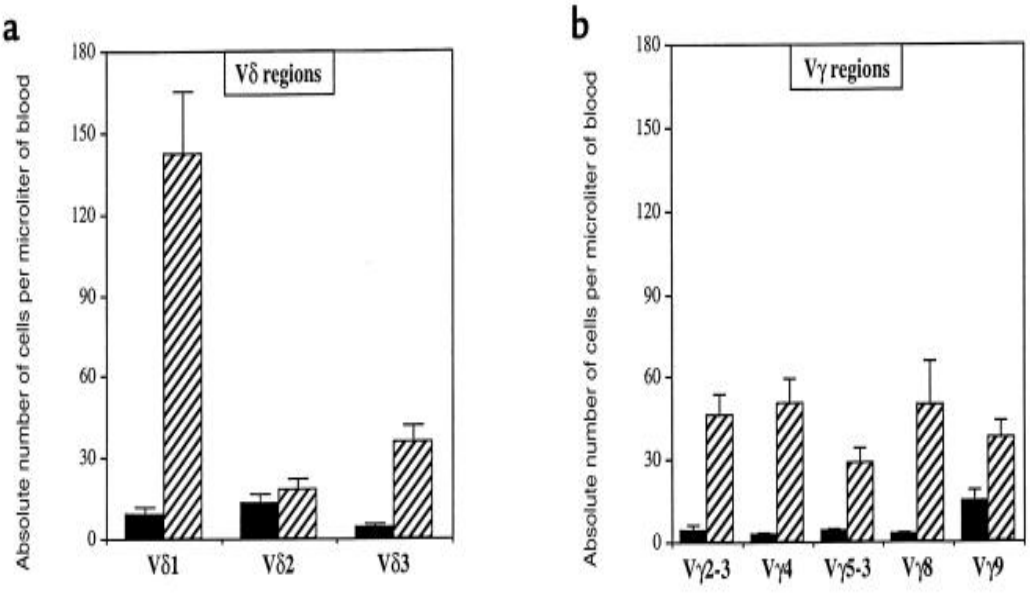


Figure 1.5: Percentage of different types of γδ T-cell expansion in CMV+ kidney recipients

This figure shows that in CMV^{pos} kidney recipients, the γδ T-cell expansion involves Vδ1+ and to a lesser extent Vδ3+ T cells. Results are expressed as mean ± SEM of absolute numbers of positive cells per microlitre of blood for each γδ T-cell subset determined in 12 CMV^{neg} patients (filled bars) and 28 CMV^{pos} patients (hatched bars). Quoted from Dechanet et al, 1999.

1.2.2.4 Initiation of anti-viral immune responses by the adaptive immune system

As mentioned in section 1.2.1, the initial immune response against viral infection starts when pathogen associated molecular patterns (PAMPs) of viral particles are recognized by the PRRs of the innate immune system. Among the most important cells of the innate immune system are professional antigen-presenting cells (APCs), which upon activation through their PRRs start to migrate to the regional lymph node (LN). During migration, APCs undergo a process of maturation in which they lose most of their ability to engulf other pathogens and develop an ability to communicate with T cells. This includes up regulation of their co-stimulatory molecules and processing of viral antigens (Takeuchi and Akira, 2010).

In the lymph node, APCs interact with naïve CD4+ and CD8+ T cells, which can recognize short viral derived peptide on MHC-II and MHC-I respectively (Wubbolts *et al.*, 1997). It has been proposed that APCs are prone to viral infection including herpesviruses, therefore, they are capable of presenting viral peptides in association with MHC-I to CD8+ T cells, see Figure 1.6. It is worth mentioning that, even if APCs are not infected they are still capable of presenting viral antigens to CD8+ T cells through a process called cross-presentation (Bevan, 2006). Furthermore, APCs can also engulf part of a virally infected cell and process it as exogenous antigens. Thus, APCs present viral peptides to CD4+ T cells through MHC-II (Klein and Sato, 2000), see Figure 1.7.

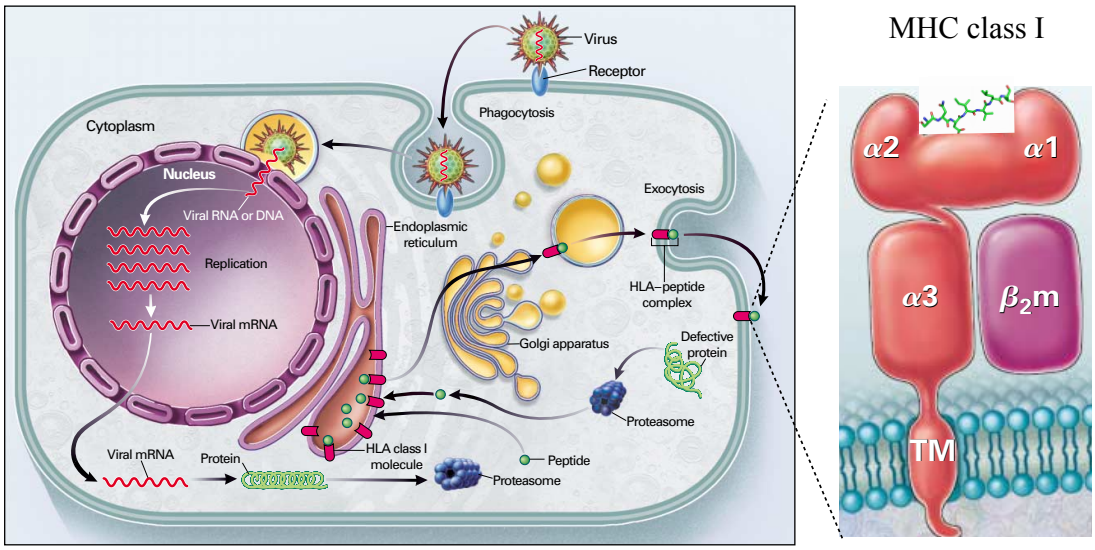


Figure 1.6: Presentation of intracellular antigen on MHC class I

The left panel demonstrates the steps of peptide processing for loading onto MHC-I molecules (**internal pathway**). Following uncoating of viral particles the viral DNA enters the nucleus and replicates within it. The viral messenger RNA (mRNA) then enters the cytosol and is transcribed into proteins. These proteins are unfolded with the help of specialized molecules called chaperones. This is followed by degrading the polypeptide chains into short peptides by proteasomes. Selected peptides are then transported into the endoplasmic reticulum, where they are loaded onto newly synthesized MHC-I molecules. Through the Golgi apparatus, the MHC–peptide complexes are then exported to the surface of the cell. In the right panel, the structure of MHC-I Molecules is shown. It consists of one light chain called Beta2-microglobulin (β_2m) and one heavy chain called α chain. The α chain has two peptide-binding domains (α1 and α2), and an immunoglobulin-like domain (α3). The α chain is connected to cytoplasm by the trans-membrane region (TM), and the cytoplasmic tail. Modified from(Klein and Sato, 2000)

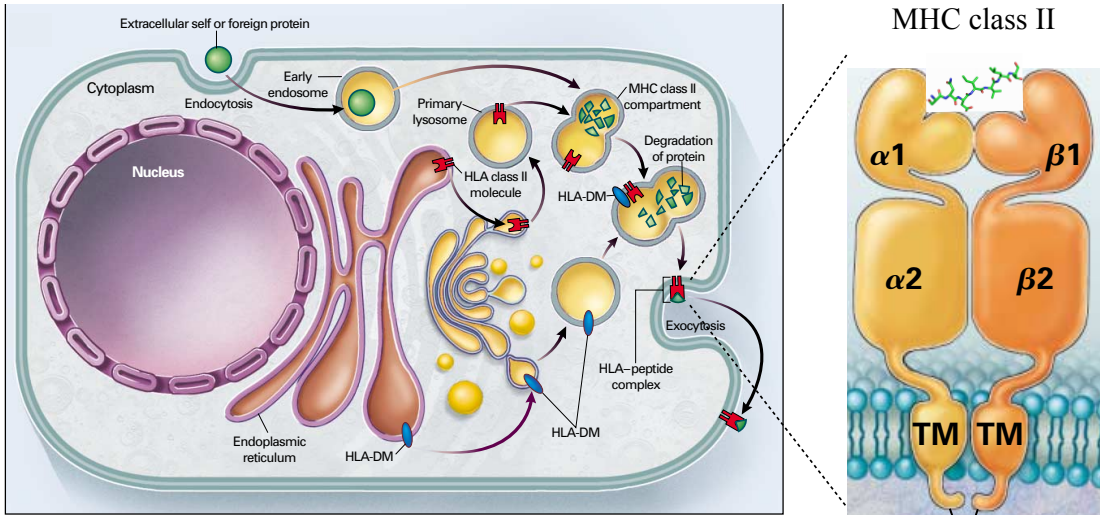


Figure 1.7: Presentation of extracellular antigen on MHC class II

This illustrates the external pathway for processing of extracellular proteins. APCs have the ability to engulf foreign proteins and sequester them into cytoplasmic vacuoles called early endosomes by an endocytic process. Simultaneously, Class II molecules, which are synthesized in the endoplasmic reticulum, are transported by way of the Golgi apparatus into primary lysosomes. Both primary lysosomes and endosomes fuse to form the MHC-II compartment. Enzymes brought into this compartment by the lysosomes degrade the engulfed viral protein into peptides. HLA-DM molecules, which are synthesized in the endoplasmic reticulum, are delivered into the MHC-II compartment by transport vesicles. The function of HLA-DM is to help in loading of the peptides onto the class II molecules. Finally, The MHC-II/peptide complexes are then exported to the surface of the cell. The right panel displays the composition of MHC-II molecules. They have two polypeptide chains; α and β chains, each chain consists of four domains; the peptide binding domain (α1 and β1), immunoglobulin-like domain (α2 and β2) and trans-membrane and cytoplasmic tail. Modified from (Klein and Sato, 2000)

1.2.2.5 T cell priming by professional antigen presenting cells

Following interaction between naïve CD4+ T cells and APCs, the CD4+ T cells undergo numerous divisions and can become polarized into distinct effector T helper (T_H) cells. In the context of herpesvirus infection most polarized CD4+ T cells will have a T_H1-type phenotype. That means they can produce large amounts of IFNγ, TNFα and IL-2 (Constant and Bottomly, 1997). Although priming of CD8+ T cells can occur in the absence of viral-specific CD4 T cells, it seems that early priming of CD4+ T cells has a crucial role in generating optimal antiviral CD8+ T cell responses (Fayolle *et al.*, 1991). For instance, an absence of CD4+ T cells has been found to compromise the formation of appropriate cytotoxic T lymphocyte (CTL) responses against herpesvirus (Smith *et al.*, 2004). In addition, chemokines that are produced following the interaction between CD4+ T cells and APCs have an important role in attraction of CD8+ T cells towards APCs (Castellino *et al.*, 2006). Moreover, interaction between CD40L of CD4+ T cells with the CD40 receptor on APCs can protect APCs from CTL-mediated death (Mueller *et al.*, 2006), which might lead to more efficient CD8+ T cell priming. Generation of specific antiviral effector CD4+ and CD8+ T cells (also called priming of naïve T cells) is illustrated in Figure 1.8.

Three models have been proposed for CD4+ T cell-mediated help to CD8+ T cells, which include the indirect model, the licence model, and the direct model. Starting with the indirect model, which is also called the classical pathway, here the main role of CD4+ T cells is to help CD8+ T cells through secretion of certain cytokines such as IL-2, which can promote expansion of CD8+ T cells. The licensing model proposes that the activation of CD8+ T cell by APCs requires prior licensing of APCs through interaction

with CD4+ T cells. In other words, activated APCs in turn can interact with CD8+ T cells, delivering help in the form of co-stimulatory signals. However, in the context of viral infection, licensing of APCs by CD4+ T cells can be bypassed. For instance, in some viral infection the APCs can be activated efficiently only through interaction between PRRs and virus receptor and thus obviating the need for CD4+ T cell licensing of APCs (Johnson *et al.*, 2009). Finally, the direct model suggests that ligation of CD40L on CD4+ T cells with CD40 on naïve CD8+ T cells plays an important role in generation of memory CD8+ T cells (Azadniv *et al.*, 2011).

In addition to the role of CD4+ T cells during acute infection, it has been demonstrated that CD4+ T cells are crucial for developing memory CD8+ T cells. This role is attributed to the ability of CD4+ T cells to down-regulate some death regulatory legend/receptor on the CD8+ T cells. Two examples are down regulation in the expression of TNF-related apoptosis-inducing ligand (TRAIL) (Janssen *et al.*, 2005) and programmed cell death protein 1 (PD1) (Fuse *et al.*, 2009) .

Contrasting with αβ T cells, as it was mentioned early in section (1.2.2.3), the exact triggering molecules and mechanism of γδ T cell activation is still an area of intense research. Nevertheless, it is believed that γδ T cells do not seem to require antigen processing and MHC presentation of peptide epitopes, although they can recognize non-classical MHC in the absence of conventional antigenic peptides (Weintraub *et al.*, 1994). Furthermore, there is other clear formal evidence that, in contrast to recognition of MHC-bound peptide antigens by αβ T cells, human γδ T cells can recognize naturally occurring small non-peptide antigens (Tanaka *et al.*, 1995).

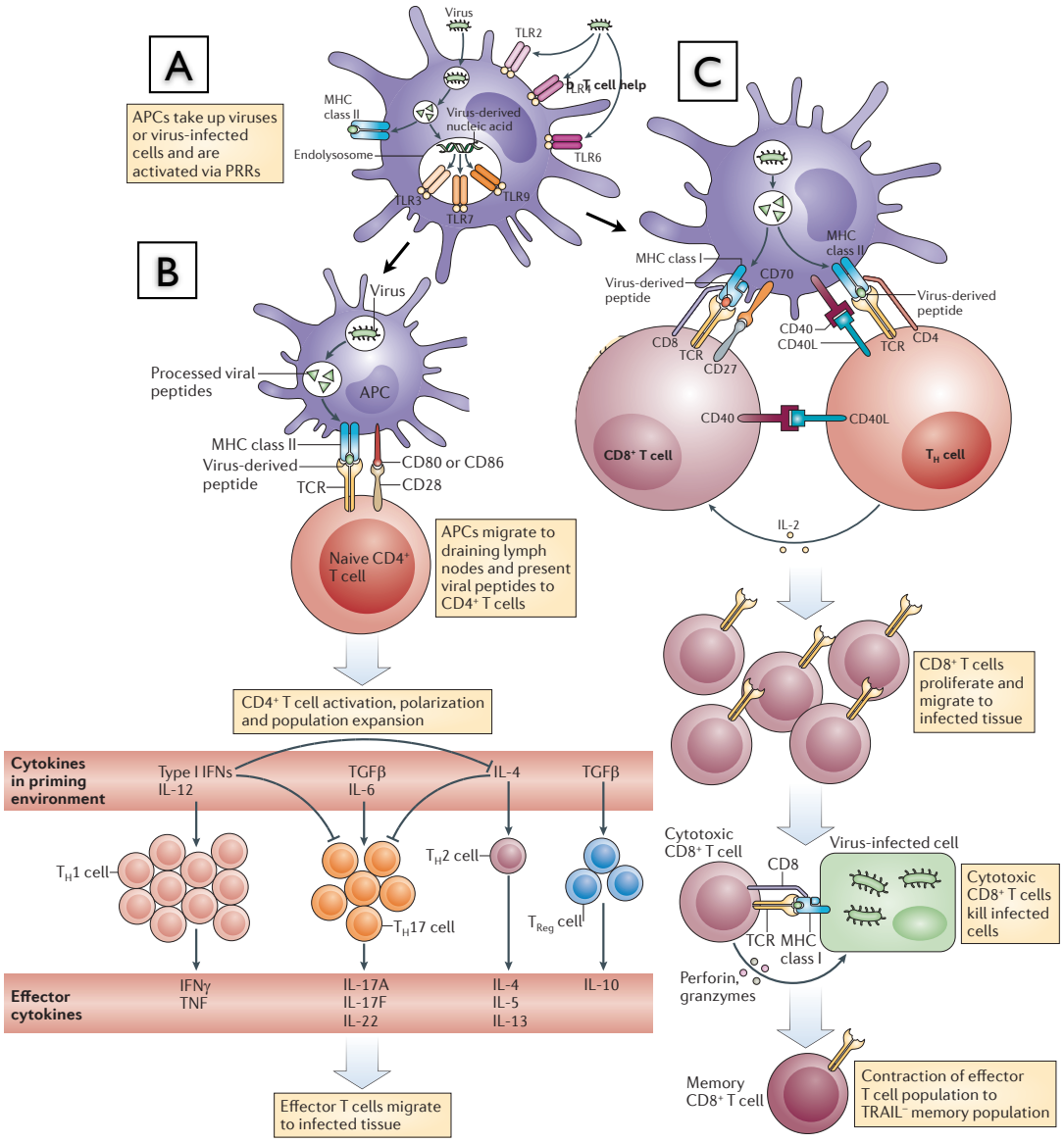


Figure 1.8: Generation of antiviral T cell responses

(A) Uptake of viral antigens by antigen-presenting cells (APCs) in infected tissue, followed by activation of APCs through pattern-recognition receptor (PRR) ligation, and the migration of these cells to draining lymph nodes. (B) Recognition of antigen-bearing APCs by naive CD4+ T cells results in the generation of T_H1 cells owing to the presence of type I interferons (IFNs) and IL-12. However, T_H17, T_H2 and regulatory T (T_{Reg}) cell populations may also be generated. (C) CD4 T cell-mediated help in the generation of CD8+ T cell effectors involves the provision of interleukin-2 (IL-2) and the activation, known as 'licensing', of antigen-presenting cells (APCs) via CD40L–CD40 interactions. Figure modified from (Swain *et al.*, 2012).

1.2.3 Immunodominance during herpesvirus infection

Priming of T cells is followed by marked clonal expansion of viral-specific CD8+ T cells that acquire different effector functions, to eliminate the virus-infected cells. This is followed by a contraction phase, in which more than 90% of expanded clones will undergo apoptosis and persistent cells will form the memory pool (Bousso and Robey, 2003). This initial expansion of viral-specific CD8+ T cells has wide diversity in terms of epitope specificity. However, an ordered hierarchy can occur as some epitopes provoke more abundant responses compared to other epitopes which give subdominant responses (Kedl *et al.*, 2003). During a re-infection with the same virus or reactivation of latent virus there will be an expansion of effector cells from the memory pool, which involves rapid expansion of the cell subsets that have a high affinity for specific epitopes. Similar to the primary infection, upon controlling of the second infection, certain expanded effector cells will become memory cells. Accordingly, with each viral reactivation the pool of memory cells will have higher epitope specificity and less clonal diversity. In immunology this phenomenon is known as immunodominance (Kim *et al.*, 2011), see Figure 1.9.

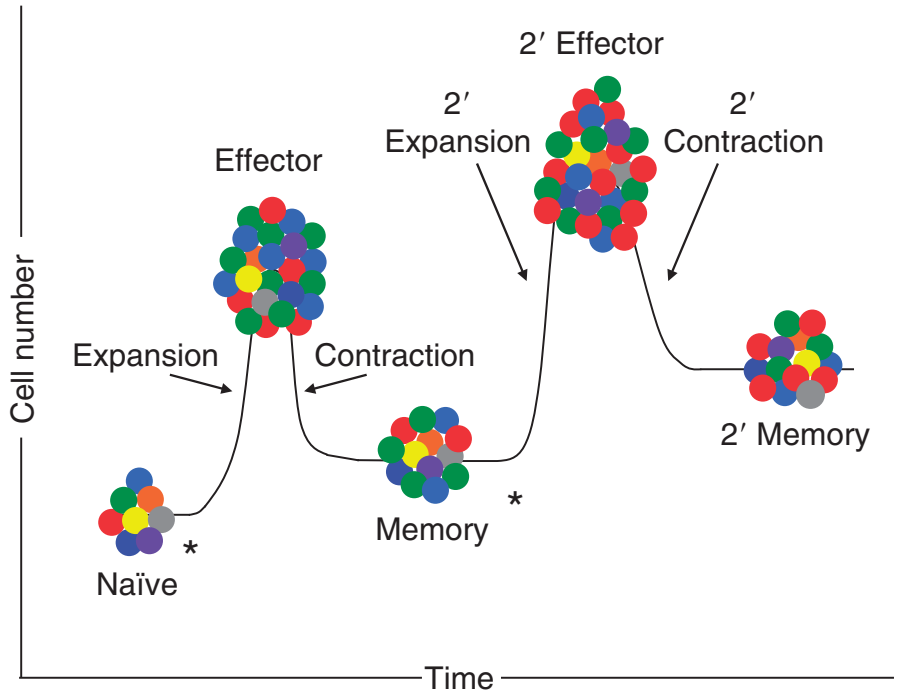


Figure 1.9: Changes in T-cell immunodominance

T cell response that is elicited during a primary herpesvirus infection consists of heterogeneous viral epitope–specific T cells. These responses are not necessarily equal and in the example depicted the T cells specific for the 'green' epitope are immunodominant following the primary infection. This pattern of immunodominance is maintained during the memory phase. However, re-exposure to the virus might result in shifting of immunodominance towards another epitope (portrayed as red dots) that has more T cell avidity. Therefore, the more frequent exposure to the same immunogenic epitope the higher memory T cell pool frequency with less heterogeneity. An asterisk indicates when exposure to the virus occurred. Figure modified from Encyclopedia of Virology, Third Edition (2008), vol. 3, pp. 73

In the context of herpesvirus infection, the immunodominant response to specific epitopes can be shifted according to the viral infection stage. For instance, in the early stage of infection (lytic cycle), the initial response will be dominated by immediate early (IE) and early (E) viral proteins. However, upon establishing viral latency the response will be shifted to latent (L) viral antigens. In other words, the herpesvirus's replication cycle is associated with expression of a wide range of viral proteins that includes; immediate early (IE), early (E) and late (L) proteins. Though the T cell response is mainly attributed to recognition of IE-1 proteins, there are other multiple proteins that can be targeted by T cells throughout the virus replication cycle. For instance pp65, which is one of the structural tegument proteins of CMV, is a major target for both CD4+ and CD8+ T cells where it is believed to be responsible for high percentage of the total CD8+ T cell antiviral response (Wills *et al.*, 1996a, Sylwester *et al.*, 2005). Besides pp65 there are other structural proteins such as pp50, pp28, gH, and gB that are thought to be of high importance in activation of T cells in the early stages of infection (Elkington *et al.*, 2003). See Figure 1.10 for more information about the other antigen types and their representation in activation of CD4+ and CD8+ T cells.

Contrasting with CMV, less data are available on VZV immunodominance. However, several VZV antigens have been identified as targets of CD4+ T cells compared to the partial understanding of VZV-specific CD8+ T cell specificity. For instance, during VZV early infection the immunodominant CD4 T cell response was toward immediate early antigens, such as IE62, IE63, ORF4, and ORF10, while in latency it shifted to late antigens that included gB, gC, gI, and gH surface glycoproteins (Arvin *et al.*, 1986, Bergen *et al.*, 1991, Arvin *et al.*, 2002).

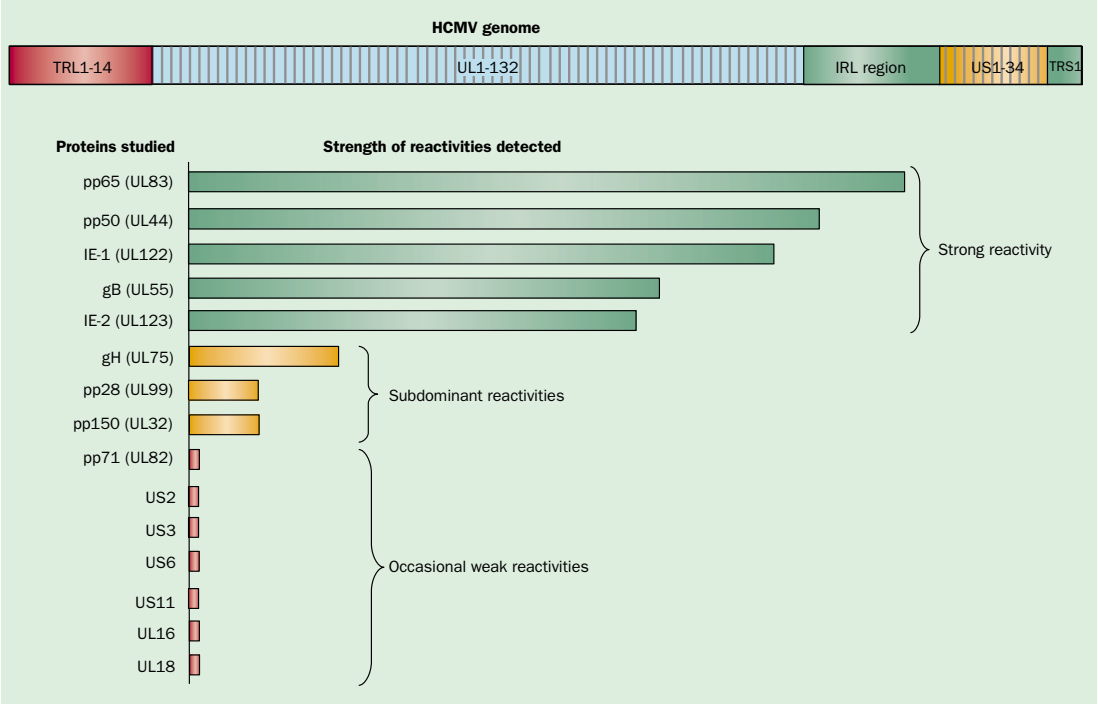


Figure 1.10: Strength of CD8+ and CD4+ T cell responses against cytomegalovirus antigens

This figure illustrates a schematic representation of the strength of CD8 and CD4 T cell responses against cytomegalovirus antigens in healthy virus carriers. Relative reactivity for each protein (shown on the left axis) is indicated on the basis of strength of T cell responses (Gandhi and Khanna, 2004).

1.2.4 T cell phenotyping

One feature that characterises cell-mediated immunity is the diversity in the function and phenotype of T cells. Moreover, even T cells specific for the same epitope might have this diversity. Several phenotypic schemes have been proposed to link certain cell function to specific phenotype. In this thesis, the combination of isoform of CD45R with a mixture of cell-surface markers such as homing receptor, co-stimulatory, and activation markers were adopted.

Generally, naïve T cells are characterized by expression of CD45RA, CC-chemokine receptor 7 (CCR7), the lymph-node homing receptor CD62L (L-selectin) and co-stimulatory molecules CD27 and CD28 (Hamann *et al.*, 1997). In contrast, memory T cells can be identified by expression of CD45RO (or absence of CD45RA) with heterogeneous expression of other markers (Klein and Sato, 2000) such as high levels of Lymphocyte function-associated antigen 1 (LFA-1; CD11a-CD18) (Okumura *et al.*, 1993). Furthermore, memory T cells can be subcategorized into different cell subsets. For instance, memory cells expressing CD45RO will be termed 'central memory' (T_{CM}) if they express CCR7 as well. Their name comes from the ability of this cell subset to respond to secondary lymphoid tissue chemokine (SLC; CCL21), and thus migrate centrally towards lymph nodes. However, CD45RO+ cells with a CCR7- phenotype express other kinds of chemokine receptors and adhesion molecules that allow them to migrate to the site of infection (Klein and Sato, 2000). In addition, CD45RO+ CCR7- memory T cells have a superior ability to secret cytokines, particularly IFNγ, and thus they are termed effector memory T cells (T_{EM}). Furthermore, according to the combined expression of CD27 and CD28, CD8+ T cells can be classified into early memory

(CD27+ and CD28+), intermediate memory (CD27- and CD28+), and late memory (CD27- and CD28-) subsets (Appay *et al.*, 2002).

In addition to surface markers, the expression of perforin and granzyme B is used to identify effector cell subsets. Contrasting to effector cell subsets generated during acute infection, which also express perforin and granzyme B, effector memory cells have a CD27- and CD28- phenotype.

It is important to mention that CD45RA can be re-expressed at a later stage of differentiation. This cell subset is called Effector Memory CD45RA Expressing T Cell (T_{EMRA}**)**. For instance, in CMV chronic carriers CMV-specific CD8+ T cells have a CCR7-, CD27-, CD28-, and CD45RA+ phenotype (Gobeil and Leib, 2012). Interestingly, this phenotype is also seen in total CD8+ T cells and is correlated to CMV-seropositivity but not that of other herpesvirus infections. Figure 1.11 demonstrates phenotypic changes in CMV specific CD8+ T cells from acute to latent infection.

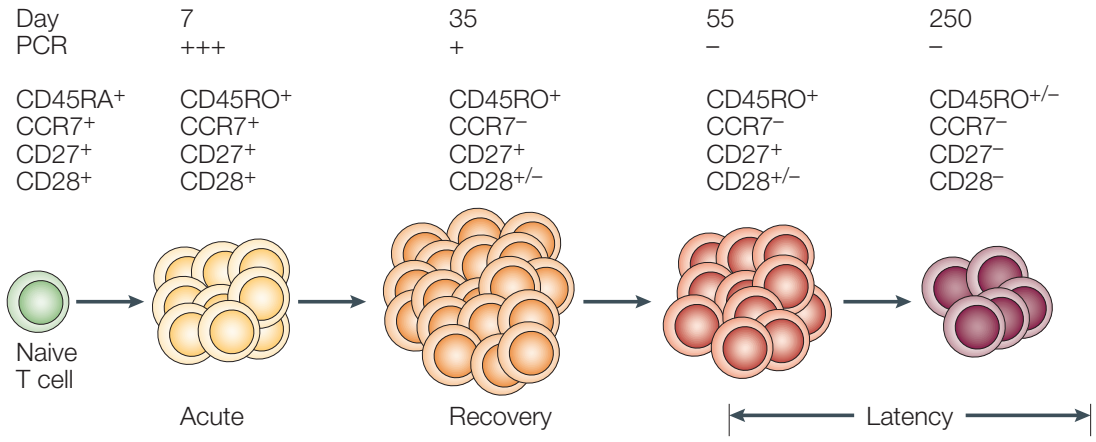


Figure 1.11: Phenotypic changes of CMV-specific T cells from acute infection to the latent state.

It shows a schematic representation of the steps of CMV-specific CD8 T-cell development. T-cell differentiation during infection with virus is characterized by the successive loss of expression of CD45RA, CC-chemokine receptor 7 (CCR7), CD27 and CD28, followed, in the recovery phase, by re-expression of CD45RA. Note, PCR indicates viral load that was measured by polymerase chain reaction. Figure reproduced from (van Lier *et al.*, 2003).

1.2.5 Immunological burden of herpesviruses infections (CMV and VZV)

So far, it is clear that the control of these viruses necessitates contributions from almost all arms of the immune system. Therefore, frequent reactivation of latent viruses would be associated with frequent activation of the immune system, which, indeed, can be a major burden to the immune system. The ability of these viruses to establish latent infection is still an area of potential research. However, the ability of these viruses to evade recognition by the immune system has been proposed as a key factor in their ability to remain latent. How CMV and VZV can evade various parts of the immune system will be discussed in the following sections.

1.2.5.1 Evasion of the immune system by CMV

Despite CD8+ T cells recognizing many CMV antigens, CMV has adopted strategies of avoiding discovery by CD8+ T cells. The key strategy to achieve this is by preventing MHC-I expression via several different mechanisms, more details are shown in Figure 1.12. The question here is that if CMV has capabilities for evading CD8+ T cells by preventing MHC-I expression then it would be reasonable to argue that CD8+ T cells play only a minor role, if not at all, in immunity to CMV. However, clear evidence against this claim comes from the clinical observations in humans and experiments in an animal model of CMV, murine CMV (mCMV), where it has been confirmed that depletion of CD8+ T cells is associated with a remarkable increase in morbidity after mCMV infection (Wills *et al.*, 1996a, Sester *et al.*, 2002b).

In other words, it is beyond doubt that CD8+ T cells are very important in CMV immunity. Therefore, regardless of the ability of CMV to avoid detection, the immune system, particularly CD8+ T cells, has ways to counter this evasion by CMV. Two

theories can explain this; firstly, CMV interference with the MHC-I complex is not absolute. The first evidence is based on the cross-priming phenomenon. This involves dendritic cells, which are professional antigen presenting cells (APCs) that capture antigens from infected cells in tissues, and then migrate to lymph nodes and present them to CD8+ T cells as exogenous antigens on MHC-I molecules. Further support for overcoming viral evasion tactics comes from the knowledge that MHC antigen presentation can be enhanced by type I (IFNα and IFNβ) and type II interferon (IFNγ), which are produced in high concentration by infected cells and lymphocytes respectively. It is believed that the IFNγ induces increased expression of MHC proteins that overwhelm the viral inhibition (Geginat *et al.*, 1997, Hengel *et al.*, 1998). Therefore, the immune system can counter some of the evasion mechanisms of CMV that has developed to evade T cell recognition (Tabi *et al.*, 2001).

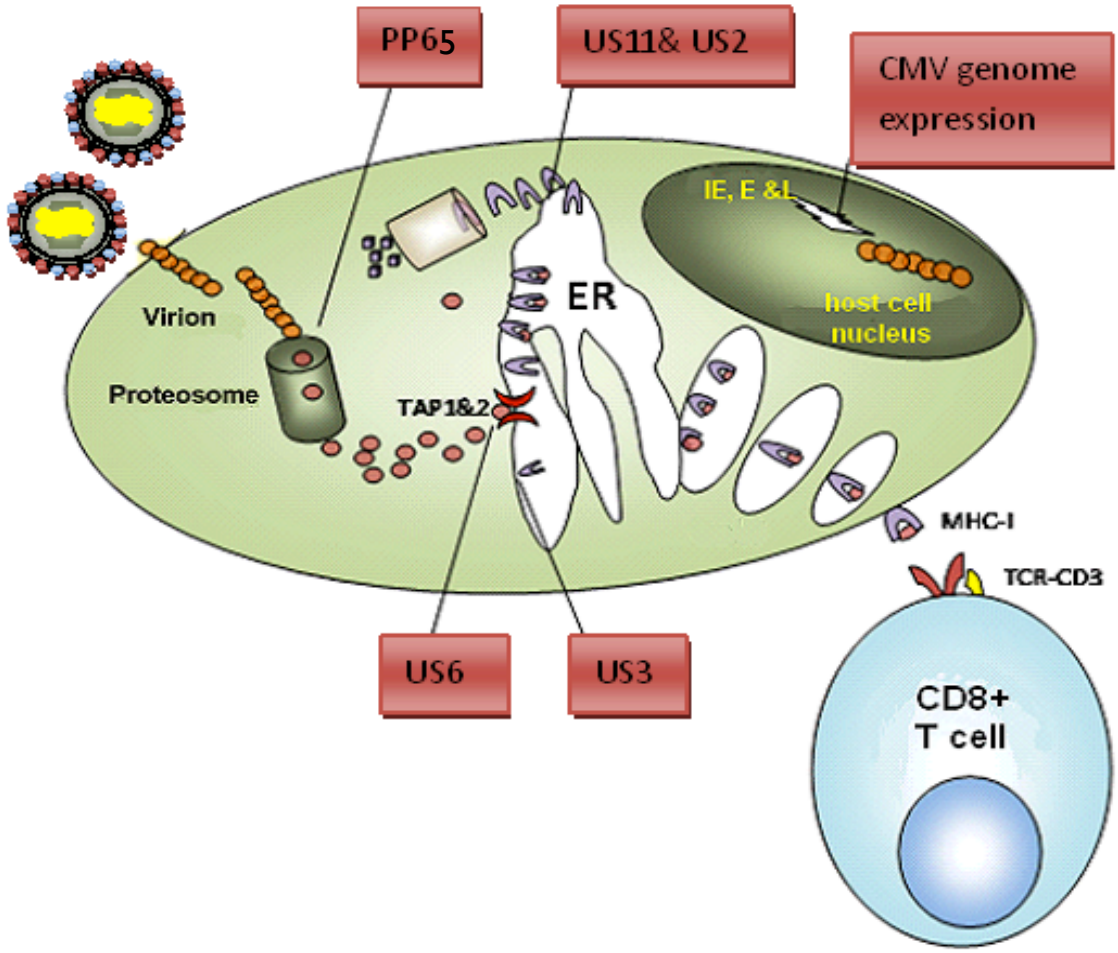


Figure 1.12: Evasion of CD8+ T cells by CMV

This figure illustrates the strategies CMV uses to evade recognition by CD8 T cells. Immediately after virus particles enter the cytoplasm of the host cell, a tegument glycoprotein (pp65) phosphorylates newly formed immediate early proteins such as IE-1 and thus protects them from being degraded by cytoplasmic proteasomes (Gilbert *et al.*, 1996). Thereafter, the expression of the CMV encoded US6 protein occurs. US6 binds the TAP complex at lumen, and prevents translocation of other processed CMV antigens into the endoplasmic reticulum (ER), where newly synthesised MHC molecules are located. Another mechanisms of interference with MHC-I expression includes retaining of newly synthesized MHC-I heavy chain inside the ER as result of US3 binding (Lehner *et al.*, 1997). Furthermore, CMV proteins US2 and US11 induce both components of MHC-I (the retained MHC heavy chain and B-2 microglobulin) to be transported to the cytosol where they are degraded by the proteasome (Wiertz *et al.*, 1996a, Wiertz *et al.*, 1996b).

Indeed, the ability of CMV to down regulating MHC-I renders the cell impervious to T cell-mediated immunity. Nevertheless, loss of MHC-I would deprive these cells of the inhibitory effect of MHC-I/NK inhibitory receptors and render these cells vulnerable to NK cell mediated lysis (Abendroth and Arvin, 2001, Eisfeld *et al.*, 2007, Wilkinson *et al.*, 2008) Moreover, expression of CMV UL111a during latency, which is a viral homologue of IL-10, has been demonstrated to down regulate expression of MHC-II molecules (Cheung *et al.*, 2009).

To escape this susceptibility to NK cell lysis, CMV has a number of viral gene products that block NK cell mediated recognition and thus cell lysis (Wilkinson et al., 2008). Two main strategies are used for this purpose. One of these strategies is through blocking NK cell stimulating receptors via an increase in the expression of cellular HLA-E through the action of the CMV UL40 protein (Tomasec et al., 2000). HLA-E is a non-classical MHC-I molecule that in normal conditions binds a restricted set of peptides derived from the leader sequences of classical MHC-I (Tomasec et al., 2000). However, the presence of CMV US6 protein prevents entry of these leader sequences of classical MHC-I to the endoplasmic reticulum (ER) and therefore also prevents expression of HLA-E (Lehner et al., 1997). Indeed this situation does not suit survival of CMV, so to overcome this, CMV UL40 signal peptide (SP^{UL40}) contains a nonameric sequence with exact sequence identity to an endogenous HLA-E–binding peptide. Therefore, it can led to upregulate HLA-E independently of TAP function (!!! INVALID CITATION !!!), and thereby, it gives a false signal (via the inhibitory receptor CD94/NKG2A) to NK cells that the cell is healthy. Very recent Prod'hommes' group has validated this model and gained insight into the molecular processes involved (Prod'homme *et al.*, 2012).

Another way to stimulate the inhibitory receptors of NK cells is via expression of UL18, which is an MHC-I homologue. UL18 was demonstrated to bind NK cell inhibitory receptor LIR1 with 1000-fold higher affinity than HLA-I molecules (Willcox *et al.*, 2003). Nonetheless, Prod'homme *et al* showed that while the expression of UL18 can inhibit $LIR1^{pos}$ NK cells, it also could stimulate $LIR\text{-}1^{neg}$ NK cells (Prod'homme *et al.*, 2007). This study might imply an additional $LIR\text{-}1^{neg}$ independent interaction in which UL18 expression promotes NK cell recognition.

Another strategy in evading NK cells is by preventing the activation of NK cell stimulatory receptors. For instance, UL16 is found to interfere with expression of some host molecules such as MICB (MHC class I polypeptide-related sequence B), ULBP1 (UL16 binding protein 1) and ULBP2 (UL16 binding protein 2), which are known to be potent NK cell stimulatory ligands. Thus, CMV prevents the NK cell stimulatory receptors from binding to their ligands (Dunn et al., 2003, Rolle et al., 2003). In addition, UL142 was found to downregulate cell surface expression of the MICA (MHC class I polypeptide-related sequence A) (Chalupny *et al.*, 2006).

Furthermore, UL141 was found to play a major role in NK cell modulation by HCMV (Tomasec *et al.*, 2005). It was demonstrated to act by preventing up regulation of ligand CD155 and CD112, which are main ligands for NK cell activating receptors CD226 (DNAM-1) and CD96 (Prod'homme *et al.*, 2010). See Figure 1.13.

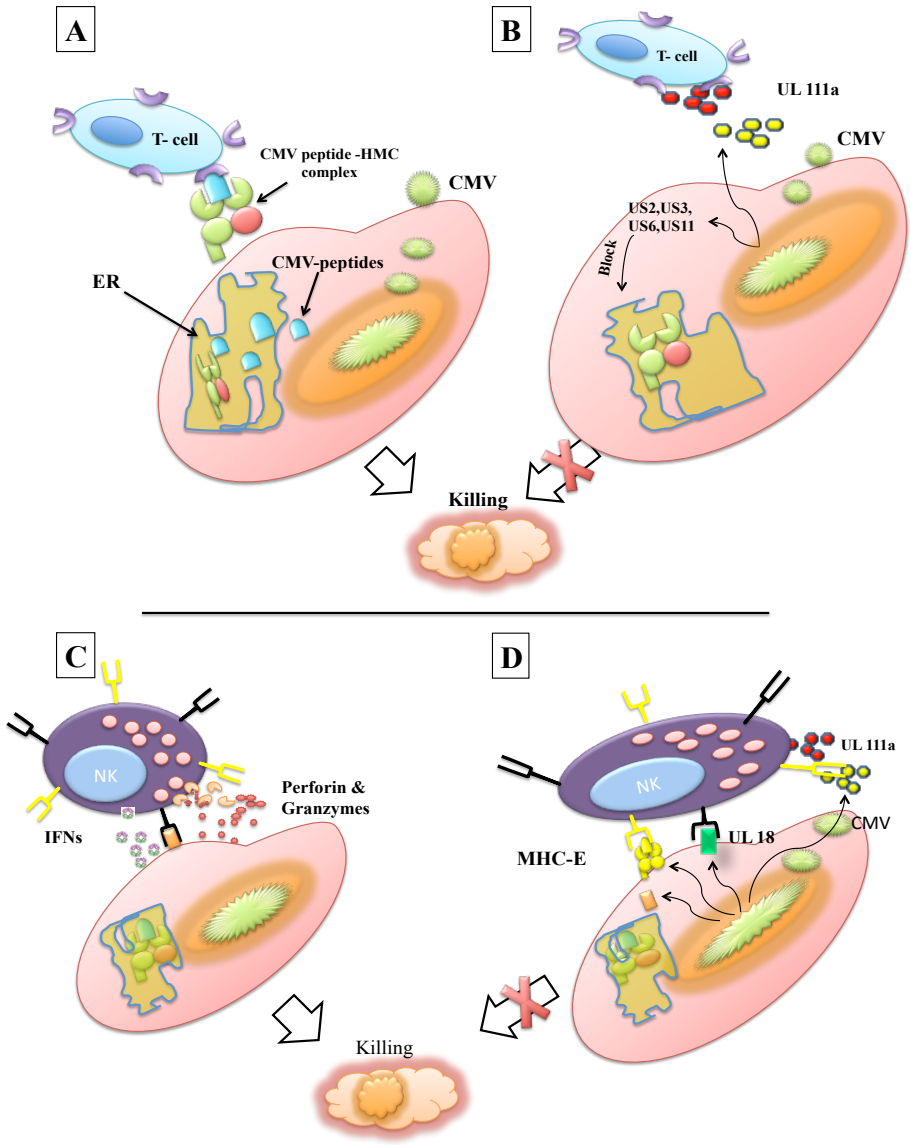


Figure 1.13: Evasion of NK cells by CMV

This illustrates mechanisms of NK cell recognition inhibition by CMV-infected cells. Typically, T cells can recognize and kill virus-infected cell (A). However; CMV encodes several genes that can block the expression of MHC-I (see Figure 1.12). It also secretes UL111a (IL-10 analogues), which down regulates MHC-II (B). The down regulation of MHC-I can make the infected cell susceptible to NK cell mediated lysis (C). Nonetheless, CMV uses the host HLA-E pathway to inhibit NK cells via the CD94/NKG2 inhibitory receptor. It also expresses a viral homologue of cellular MHC Class I, UL18.

1.2.5.2 Evasion of the immune system by VZV infection

As explained early in section 1.2.1, the innate immune system plays a major role in protection against herpesvirus infection. This role contributes to the establishment of an antiviral state in non-infected cells through secretion of a variety of interferons and cytokines (Muller *et al.*, 1994, Sadler and Williams, 2008). VZV has two strategies to prevent this antiviral response. The first strategy is by inhibiting expression of IFNα, through expression of ORF63 (Ambagala and Cohen, 2007). The second strategy is by impairing the antiviral signaling of IFNγ that might involve ORF66 (Schaap *et al.*, 2005). The exact mechanisms by which these molecules can modulate the immune system are not fully understood.

In addition, ORF66 seems to be important in down-regulation of MHC-I expression via preventing MHC-I transportation to the cell surface by the Golgi apparatus (Abendroth *et al.*, 2001). Furthermore, VZV encodes an immune-modulatory function, which can directly interfere with MHC-II expression at the transcription level (Abendroth *et al.*, 2000). This effect seems to be due to inhibition of IFNγ expression (Black *et al.*, 2009).

The interactions between VZV infected cells and NK cell receptors have not been extensively studied. For instance, how VZV can evade recognition by NK cells as a consequence of MHC-I down regulation on infected cell surfaces via the viral protein kinase ORF66 have not been demonstrated (Eisfeld *et al.*, 2007). Likewise, to the best of our knowledge, no mechanisms of VZV interference with NK cell activating ligands have been reported.

1.2.6 Studies on CMV-specific immune responses

The influence of cytomegalovirus carrier status on lymphocyte subsets and natural immunity was noticed since the late eighties (Gratama et al., 1987). In this context there are several studies that confirm the importance of CD8+ T cells in controlling CMV disease, particularly in primary infection. For instance, subsets of CD8+, CD57+ cells in normal, healthy individuals were found to correlate positively with human CMV carrier status, phenotypic and functional analyses (Wang *et al.*, 1993, Wang and Borysiewicz, 1995). Furthermore, Sester and colleagues demonstrated that during primary infection, the cellular immune response is mainly dominated by CMV-specific CD8+ T cells (Sester *et al.*, 2002b). This was also established by Reusser's group, who showed that the delay in restoration of CMV-specific CD8+ T cells after bone marrow transplantation (BMT) increased the risk of developing severe CMV disease (Reusser *et al.*, 1991). Furthermore, transfer of CMV-specific CD8+ T cell clones derived from the donor bone marrow led to improvement in outcome with regards to CMV infection in recipients (Walter *et al.*, 1995).

In recent years, there has been an increasing literature on the role of CD4+ T cells in controlling CMV infection. For instance, studying the characteristics of CMV-specific CD4+ and CD8+ T cells during primary CMV infection in renal transplant recipients demonstrated that formation of CD8+ T cells alone was not sufficient for controlling infection; effector memory CD4+ T cells were shown to be essential for recovery of CMV infection (Gamadia *et al.*, 2003). This is also true in the case of BMT patients, where reconstitution of cellular immunity against CMV required both CMV-specific CD8+ and CD4+ T cells (Walter *et al.*, 1995). Further evidence of the importance of

CD4+ T cells in defence against CMV is the ability of CMV-stimulated CD4 cells (from CMV-seropositive but not CMV-seronegative donors) to suppress CMV gene expression in CMV encephalitis (Cheeran *et al.*, 2000).

It is believed that the chief function of CD4+ T cells is to give help to CMV-specific CD8+ T cells, particularly in maintaining the memory response. The initial suggestion that CD4+ T cells are playing this important role comes from the observation that cytotoxic CD8+ T cell activity declined in patients deficient in CD4+ T-helper cells specific for CMV, which indicates that CD4+ helper cell function is essential for the persistence of CD8+ T cells (Walter *et al.*, 1995). However, the murine model of CMV infection suggests this role is only important during the primary phase of infection; in other words, the depletion of CD4+ T cells during recall responses has minimal or no effect on the memory CD8+ T cell response, meanwhile depletion during the priming phase (acute stage) is associated with a remarkable reduction in responses by memory CD8+ T cells to re-infection (Shedlock and Shen, 2003).

Besides giving help to CD8+ T cells, CD4+ T cells can directly kill CMV-infected cells. It is proposed that in primary CMV infection, cytotoxic CD4+ T cells appear after cessation of the viral load. These cells are characterized by a loss of CD27 and loss of CD28, and upregulation of CD57, which indicates an advanced stage of cellular differentiation. They also have cytolytic molecules such as granzyme B and perforin, which might be used in killing of CMV-infected cells (Appay, 2004). The triggering of killing potential of these CD4+ T cells can be via direct recognition of endogenous antigen in addition to exogenous antigens (the normal pathway for CD4+ T cell activation), as shown by studies of the CMV-gB protein, which is processed and

expressed efficiently by MHC-II of the infected cells (Hegde *et al.*, 2005). Although the response of CD4+ T cells can be demonstrated against a wide range of CMV antigens, the main responses are found to be against pp65 and glycoprotein B, which are also strong antigens for CD8+ T cells. IE1, though a strong antigen for CD8+ T cells, is less stimulatory than pp65 and gB for CD4+ T cells (Sylwester *et al.*, 2005). This may be because IE-1 does not access the class II processing pathway as readily as pp65 and gB, which are both structural components of the virus particle and more classical exogenous antigens.

Furthermore, Casazza et al showed that acquisition of direct antiviral effector functions by pp65-specific CD4+ T cells with cellular maturation (Casazza *et al.*, 2006). This included acquiring a highly differentiated effector memory phenotype, that was characterized by the relatively low frequency of IL-2–producing CD4+ T cells compared with IFN-γ, TNF-α, and MIP-1β production. Indeed, this suggests that the primary role of CMV pp65-specific CD4+ T cells during chronic infection is not solely one of supplying CD4+ T cell help.

Although it seems that the cooperation between NK cells on one hand, and B cells and αβ T cells on the other hand are sufficient to prevent most infections, γδ T cell deficiency is found to be associated with immune deficiency (Chung *et al.*, 2006). Besides that, a remarkable expansion of γδ T cells can be demonstrated during bacterial as well as some viral and parasitic infestation. For instance, γδ T cell proportions increase considerably following infection with intracellular pathogens such as Mycobacterium tuberculosis (Janis *et al.*, 1989), influenza (Carding *et al.*, 1990), Epstein Barr virus (De Paoli *et al.*, 1990), and Plasmodium (Worku *et al.*, 1997).

Furthermore, clinical studies in transplant recipients showed that CMV infection resolves better when circulating γδ T cells expand (Dechanet *et al.*, 1999b). Based on these and other observations that indicate the presence of interaction between different T cell subsets (Vincent *et al.*, 1996), there is a strong belief that γδ T cells play a very important role in immune pathology of infectious disease including CMV infection. The percentage of $V\delta2^{neg}$ γδ T cells in peripheral blood has ben shown to remarkably increase in renal transplant patients with active CMV infection (Dechanet *et al.*, 1999b). Moreover, the resolution of CMV viremia was found to be directly proportionate to the expansion of the $V\delta2^{neg}$ γδ T cell population (Lafarge *et al.*, 2001). Indeed, these observations strongly indicate the important role of $V\delta2^{neg}$ γδ T cells in combating CMV infection. Supporting this proposal, Halary and colleagues generated γδ T cell clones from renal transplant patients (Halary *et al.*, 2005). They demonstrated that $V\delta2^{neg}$ γδ T cells specifically react with CMV-infected cells by producing the pro-inflammatory cytokine, tumour necrosis factor –α (TNFα).

1.2.7 Influence of CMV chronic infection on the immune system of elderly subjects

As discussed above, the frequent reactivation of CMV latent infection can be a major burden on the immune system. In recent years, there has been an increasing interest in latent CMV infection as a key factor in immunosenescence.

By definition, the term 'immunosenescence' refers to the steady decline of immune system function by natural age advancement. This includes deterioration in both the capability to fight infections and ability to detect and eliminate transformed cells, in addition to poor development of long-term immune memory (Koch *et al.*, 2007).

Therefore, it is considered as a major contributor to high morbidity and mortality among the elderly. Although immunosenescence can be attributed to an aging process that naturally affects most of the body's systems, other factors such as continuous challenging of the immune system by different antigens might play a very important role in accelerating this phenomenon (Franceschi *et al.*, 2000).

Although the effects of immunosenescence involve all parts of the immune system, this review focuses mainly on alterations of the adaptive immune system, particularly T cell function, because functions of B cells depend on an appropriate function of helper T cells (Marc E. Weksler, 2002). Although most functions of the innate immune system, including antigen presentation by APC, are well preserved in the elderly, it should be emphasized that the innate immune system is also affected by aging associated changes. For instance, mortality and morbidity can be predicted according to NK cell status (Solana *et al.*, 2006). Furthermore, elevation in pro-inflammatory factors in the elderly is partially attributed to the innate immune system ageing (Franceschi *et al.*, 2007). The main differences in immune phenotype between young and elderly are shown in Figure 1.14.

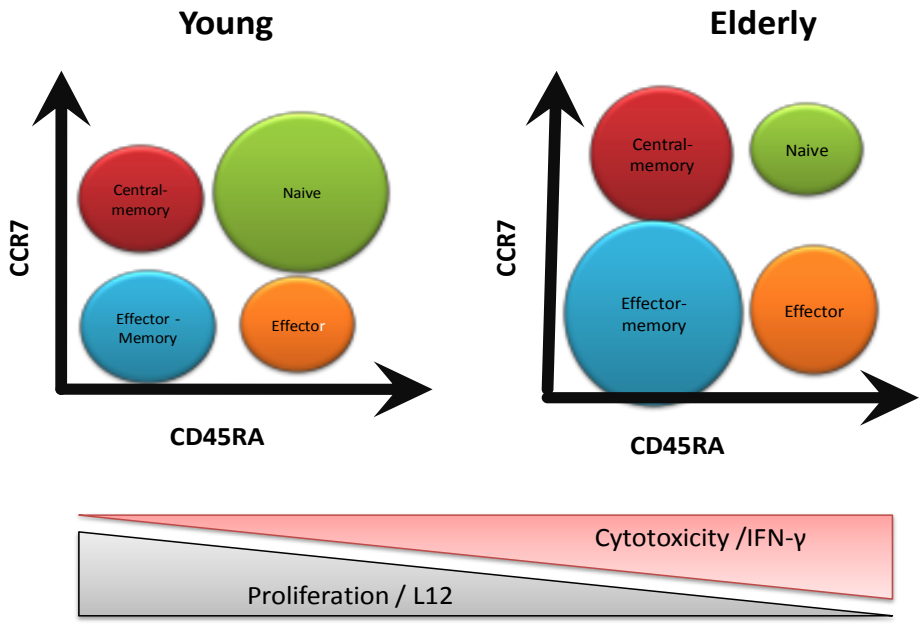


Figure 1.14: Changes in the proportions of Naive and memory T cells with ageing

Summary of changes in proliferation capacity, immune-phenotype and cytokine profile with advancing age. Homing chemokine receptor (CCR7) and phosphatase isoform (CD45RA) were used to determine the differentiation status of lymphocytes, the less expression of CD45RA/ CCR7 the higher the differentiation. While low differentiation status is associated with high proliferation capacity, the cytotoxic function (measured by IFNγ production) is remarkably increased in highly differentiated cells. The main difference between elderly and young seems to be the size of the naive and effector memory cell pools. Modified from(Koch *et al*., 2007).

1.2.8 Explanation of the Immune Risk profile in context of CMV infection

A large body of data demonstrates the contribution of chronic CMV infection to immunosenescence (Looney *et al.*, 1999, Khan *et al.*, 2002a, Ouyang *et al.*, 2003a, Ouyang *et al.*, 2003b). For instance, besides the clonal expansions of CD8+ T cells that are characteristically associated with chronic CMV infection, the phenotypic changes in these cells are found to be very similar to those associated with age-related alterations (Looney *et al.*, 1999, Olsson *et al.*, 2000), which indeed emphasises the impact of CMV on the ageing of the T cell immune system.

One of the best known studies that addressed the changes in the immune system with age advancing is the Sweden Octogenarian longitudinal study (OCTO) (Olsson et al., 2000). The result of this longitudinal study suggests a set of immunological parameters as an indicator of morbidity and mortality in healthy elderly subjects (≥85 years). These parameters include: low numbers of naive T cells with remarkable reduction in the diversity of the T cell receptor (TCR) repertoire and an increase in the absolute numbers of memory T cells. There is also an inversion in the CD4: CD8 T cell ratio with a low number of B cells. Recently, CMV seropositivity has been added to these criteria, and this is known as Immune Risk profile (IRP), see Figure 1.15.

Interestingly, the contribution of CMV to this IRP seems to exceed the anticipation. For example, the inverted CD4:CD8 ratio in this profile can be explained by continuous challenging of T cells by reactivation of CMV until they reach replicative senescence (proliferation exhaustion). At this stage, CD8+ T cells become apoptosis resistant while CD4+ T cells become more susceptible to apoptosis (Pawelec *et al.*, 1996, Effros and

Pawelec, 1997). Therefore, CD8+ T cells accumulate, CD4+ T cells decrease and the CD4: CD8 ratio becomes inverted. Support for this notion is that the clonal expansion of T cells is found to be predominantly of CMV specific CD8+ T cells (Khan *et al.*, 2002b, Khan *et al.*, 2004). It is worth noting that the majority of these cells are non-functional. However, the high absolute number of these cells could be the cause of an imbalance in the cytokine production profile in the elderly (Almanzar *et al.*, 2005), see Figure 1.16.

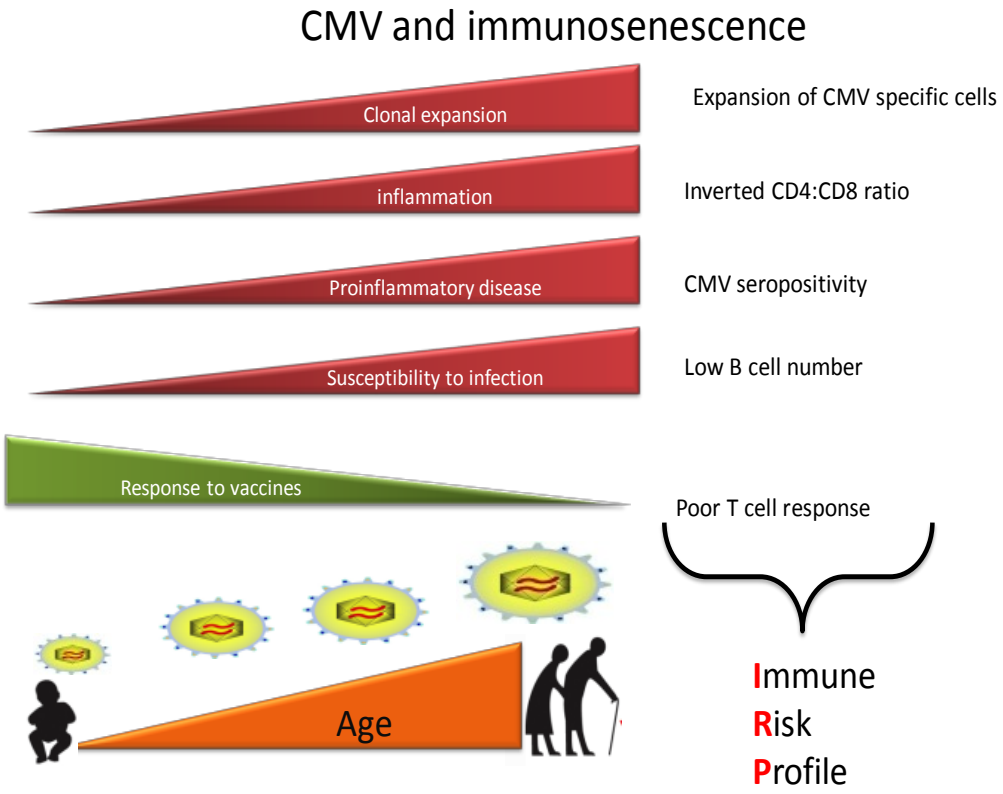


Figure 1.15: Explanation of the immune risk profile in the context of chronic CMV infection.

The increases in susceptibility to infection with a poor response to vaccines in addition to the high incidence of pro-inflammatory diseases are the main changes that occur with advancing age. Collectively these changes are called the immune risk profile (IRP). Impact of chronic CMV on the immune system such as, expansion of CMV specific CD8+ T cells with inversion of CD4:CD8 ratio and low number of B cells can be the reason behind these changes. Modified from. (Pawelec *et al.*, 2009)

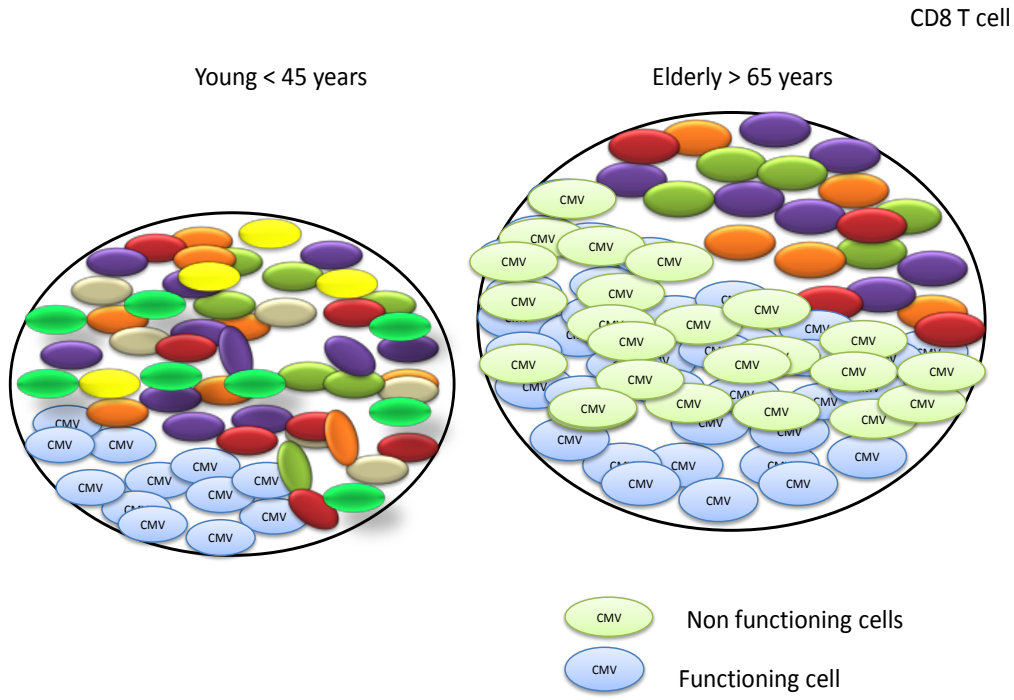


Figure 1.16: CD8 T cell memory pool in CMV seropositive healthy young and elderly donors

This demonstrates the effects of chronic cytomegalovirus infection on the size of the repertoire of both naive and memory T cells. The high lymphocytosis that is seen in the elderly can be attributed to repeated reactivation of latent CMV or even re-infection with CMV. Each time more naive T cells become CMV-specific effector and memory T cells. This will lead to a decrease not only in the naive T cell repertoire but also to accumulation of CMV-specific CD8+ T cells that are apoptosis resistant, causing inversion of the CD4:CD8 ratio and increase in pro-inflammatory cytokines that characterizes the immune profile of the elderly.

1.2.9 VZV in immunocompromised subjects

Although VZV causes self-limiting disease, this can be extremely dystrophic in certain categories of patient, such as malignancy, immunosuppressed individuals and neonates. Furthermore, the development of chickenpox in immunocompromised children is not only associated with considerable morbidity but also with a significant increase in mortality rate. The typical example where VZV has devastating sequelae is in children under chemotherapy due to cancer. For instance, the overall mortality rate from varicella in children with cancer has been reported in the late 1980's to be as high as 7% (Feldman *et al.*, 1975, Feldman and Lott, 1987). However, the subsequent introduction of therapeutic acyclovir shortens the cutaneous course of the infection and reduces the risk of dissemination and a more serious illness (Feldman and Lott, 1987, Nyerges *et al.*, 1987).

1.3 Aims and objectives

Primary infection with both CMV and VZV usually occurs at an early age, followed by lifelong persistent infection (latency phase). Until now, the precise mechanisms of herpesvirus latency are not fully understood. However, it is clear that the immune system has a crucial role in keeping replication of these viruses in check. Indeed, this immune response seems to multifaceted, with central roles for different subset of T cells (αβ and γδ T cells), besides the importance of antibody and NK cells. Indeed, frequent reactivation of latent viruses, particularly CMV, can be a major burden to the immune system. In addition, contribution of all of these arms of the immune system to just keeping these viruses in a latent state rather than eradicating them must have its impacts on the configuration of the immune system. Therefore, the main aim of this thesis was to understand the immune responses against chronic infection with these two common herpesviruses, namely CMV and VZV infections. To achieve this aim several objectives were proposed to investigate the following:

- Influences of chronic CMV carriage on the immune system of healthy subjects.
- Role of unconventional (γδ) T cells in CMV infection.
- Impact of CMV infection on the sequelae of other concurrent diseases. Here any association between CMV and ischemic heart disease (IHD) morbidity was investigated.
- Immunological defects behind the reactivation of VZV infection during acute lymphocytic leukaemia (ALL) were examined.

2 Materials And Methods

2.1 Donors

Blood samples were collected from three different donor groups. The first group involves healthy donors; these are workers in the Duncan building in the University of Liverpool. In the second group, patients with ischaemic heart diseases were recruited from the Cardiology Department, Newcastle Freeman Hospital. Meanwhile, the third group of donors consisted of children with acute lymphocytic leukaemia, who were admitted to the oncology unit in Alder Hey hospital. Since there were considerable variations among these donors groups, the characteristics of each group were discussed in more details with each related results chapter. All samples were obtained with informed consent of the donor to participate in the study. The work in this thesis was approved by Research Ethics Committee (REC). REC number for each group of donor is summarized below..

Chapter	Donors	Research Ethics Committee (REC) Number
3 and 4	Healthy	REC 2k/175
5	Patients with Ischaemic Heart Diseases	REC 09/H0905/50
6	Patients with Acute Lymphocytic Leukemia	REC 08/H1002/83

2.2 Peripheral blood mononuclear cell (PBMC) preparation

Approximately 20ml of peripheral blood was taken from each donor by venipuncture. This blood was transferred immediately into a tube containing anti-coagulant (600ul of preservative-free heparin), and then it was diluted with an equal volume of RPMI 1640 media (Sigma, UK). Each diluted sample was then divided into two equal amounts

(usually 20ml). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Ficoll-Paque^{TM} Plus (GE Healthcare Bio-sciences AB, UK). Blood was gently layered onto an equal volume of Ficoll-Paque^{TM} Plus and centrifuged at 503g (equivalent to 1800rpm) for 20 min at room temperature in a bench-top centrifuge with the brake off, see Figure 2.1.

Next, PBMC were harvested from the interface and washed twice with RPMI. In the first wash 20ml of RPMI was used (centrifugation at 1800rpm =503g for 10min with brake on 9). In the second wash, 40ml of RPMI was used at 1200rpm (=302g) centrifuge speed and brake rate 9. Then, after removing supernatants, cell pellets were resuspended in 10ml of growth medium (RPMI 1640 medium supplemented with 10% heat inactivated foetal calf serum [FCS], 2mM Glutamine, 50µg/ml Penicillin and 50µg/ml Streptomycin) and counted using a haemocytometer to calculate the number of the cells. 2ml of plasma was also removed from each separated blood sample for serological testing.

Aiming to avoid multiple bleeding of donors any extra-PBMC cell yield was preserved at -80°C in freezing media (FCS containing 10% DMSO). Each time cryo-preserved PBMC were used, they were firstly thawed, and then washed twice in FACS buffer (PBS containing 0.1% bovine serum albumin [BSA] and 2mM EDTA). EDTA was used to disaggregate any cells that were clumped or that were adherent to the plastic.

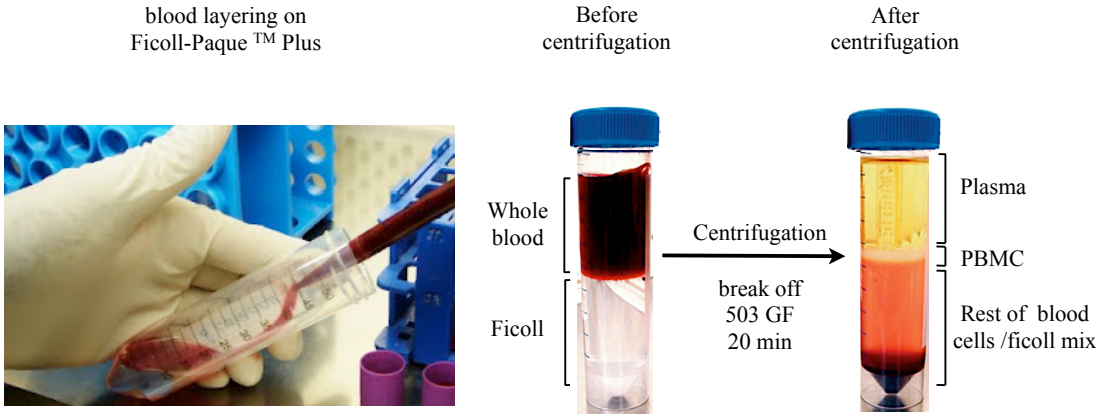


Figure 2.1: Blood layering and Ficoll Centrifugation -

It demonstrates the steps of separation of peripheral human blood by density gradient centrifugation. The two tubes demonstrate different layers before and after spin.

2.3 Serology

2.3.1 ELISA

2.3.1.1 Anti-CMV ELISA (IgG) test

Donors' plasma samples were quantitatively analyzed for anti-CMV antibody titre using a commercial cytomegalovirus (CMV) IgG ELISA kit (EUROIMMUN AG). Briefly, 1µl of plasma was diluted in 100µl sample buffer and added to micro-titer plate wells in duplicate. To create a standard curve from which the concentration of antibodies can be calculated, three calibrators of known concentration (provided by the kit) were included. In addition negative and positive control samples were also used. The plates were incubated at room temperature for 30 minutes. Plasma samples were then discarded and each well was washed 3 times with wash buffer and then incubated with 100µl of CMV antigen-enzyme conjugate for 30 minutes at room temperature. Unbound antigen was discarded and wells washed again 3 times with wash buffer. Then 100µl of chromogen/substrate was added to each well. Plates were left for 15 minutes at room temperature to allow colour development, followed by adding 100µl of stop solution. Finally photometric measurement of the colour intensity was made at the wavelength of 450 nm within 30 minutes of adding the stop solution. To determine the concentration of antibodies in plasma the Relative Unit (RU) of antibody concentration was calculated using the calibration curve, see Figure 2.2. The interpretation of results was carried out according to EUROIMMUN AG recommendation see Table 2.1.

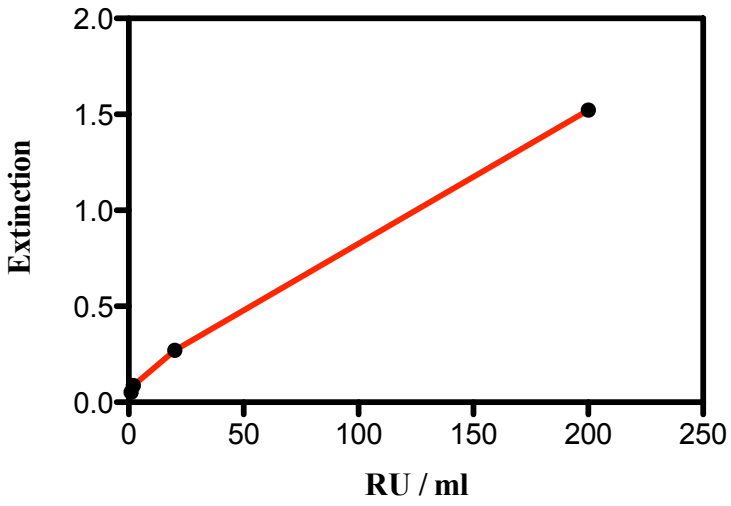


Figure 2.2: Calibration curve

The standard curve was created by point-to-point plotting of the extinction values measured for the 3 calibration sera against the corresponding units. The Y-axis indicates the extinction reading while the X-axis denotes the concentration in relative units (RU/ml).

Table 2-1: Interpretation of CMV status according to the CMV- specific antibody titer

CMV-antibody estimated concentration	CMV status
<16 RU/ml	Negative
≧ 16to<22 RU/ml	Borderline
≧22 RU/ml	Positive

2.3.1.2 Avidity determination of IgG antibodies against CMV in plasma

Identification of primary CMV infection is usually based on the detection of virus-specific IgM antibodies. However, this approach sometimes is unreliable due to persistence of high titre of IgM even in long standing infection. In addition false-positive IgM antibody tests for CMV can be found in patients with acute Epstein-Barr virus infection (Miendje Deyi *et al.*, 2000). In recent years additional determination of antibody avidity has become a proven method for differentiation between relatively fresh and long-standing infection. Initially the immune system reacts to an infection by forming low-avidity IgM antibodies. With continued disease duration, IgG that are more precisely adapted to the antigens are produced and the avidity increases. Therefore, detection of high avidity antibodies indicates that infection is at a late stage.

According to the manufacturers' protocol of the cytomegalovirus (CMV) IgG ELISA kit (EUROIMMUN) 1µl of plasma was diluted in 101µl sample buffer and added to micro-titer plate wells in duplicate. For controls, the provided negative and positive samples were used. The plates were incubated at room temperature for 30 minutes to allow antibodies to coat the well surface. Plasma samples were then discarded and each well was washed with wash buffer and then incubated for 10 minutes (room temperature) with 200µl of urea solution into each of the first micro plate wells and 200µl of phosphate buffer into each well of the second micro-titer strip. This was followed by a washing step and then addition of CMV antigen-enzyme conjugate for 30 minutes at 37°C. Unbound antigen was discarded and wells washed again 3 times with wash buffer. Then 100µl of tetramethylbenzidine (TMB) substrate was added to each well. Plates were left for 10 minutes at room temperature to allow colour development, followed by

adding 100µl of stop solution. Finally the photometric measurement of the colour intensity was made at the wavelength of 450 nm within 30 minutes of adding the stop solution.

The presence of low avidity antibodies in a donor's serum has been proved if the ELISA extinction value is considerably reduced by urea treatment. In order to quantify the results a relative avidity index (RAI) is calculated as follows:

$$\frac{\text{Extinction of sample with urea treatment}}{\text{Extinction of sample without urea treatment}} \times 100 = \text{relative avidity index (RAI) in \%}$$

The cut-off value of low avidity antibodies recommended by EUROIMMUN is 40%. Table 2-2 summarizes the interpretation value.

Table 2-2: Cut-off value of low avidity

Relative Avidity Index (RAI)	Antibodies Avidity	CMV infection
<40%	Low-avidity	Fresh infection
40% - 60%	Equivocal	Unknown
>60%?	High-avidity	Log-standing infection

2.3.1.3 Qualitative detection of IgG antibodies to Varicella-Zoster Virus (VZV)

Aiming to determine if the donors were immune to VZV, a special ELISA kit (Diamedix Immunosimplicity Is-VZV IgG EIA Test System; catalog No 720-380 - 96 Tests) was used. Basically, the protocol was very similar to that of the CMV ELISA where donors' samples were prepared by adding sample diluent at 1:101 ratio. This was followed by transferring 100µl of Calibrator, Controls and diluted samples into the antigen wells. The protocol included use of Calibrator and Controls. Also one well was reserved for reagent blank (100µl of Sample Diluent). This was followed by incubation at room temperature (18-30°C) for 30 minute. After discarding contents of the wells they were washed 3 times with Wash Solution. Next 100µl of Conjugate was added to each well and the plate was incubated at room temperature (18-30°C) for 30 minutes, followed by a wash step as above. Subsequently 100µl of Substrate Solution was added to each well and incubated at room temperature for 30 minutes. Finally 100µl Stop Solution was added to each well and the absorbance was read at 450/600-630 nm. The results were presented as EU/ml (ELISA Units/ml), which can be calculated using the following formula:

$$\text{EU/ml of sample} = \frac{\text{EU/ml of Calibrator}}{\text{Absorbance of Calibrator}} \times \text{Absorbance of sample}$$

Results can be interpreted according to the following values

Table 2-3: Interpretation of VZV –ELISA values

EU/ml	Interpretation
<15.0 EU/ml	Nonreactive (Negative) for anti-VZV IgG: Presumed non-immune to VZV
15.0-19.9 EU/ml	Equivocal No conclusion on the immunity of VZV
≧20.0 EU/ml	Reactive (Positive) for anti-VZV IgG: Presumed immune to VZV

2.3.1.4 Qualitative detection of IgG antibodies to herpes simplex virus (HSV) type 1 and /or type 2

Here results were obtained with the Diamedix immunosimplicity Is-HSV 1 & 2 IgG EIA Test System (Catalog No. 720-340 – 96 Tests) all steps of procedure were exactly the same as that for VZV ELISA mentioned above. However the interpretation of results was slightly different.

$$\textbf{EU/ml of sample} = \frac{\textbf{EU/ml of Calibrator}}{\textbf{Absorbance of Calibrator}} \textbf{ X } \textbf{Absorbance of sample}$$

Table 2-4: Interpretation of HSV –ELISA values

EU/ml	Interpretation
<16.0 EU/ml	Negative for anti-HSV 1&2 IgG
16.0-19.9 EU/ml	Equivocal for anti-HSV 1&2 IgG
≧20.0 EU/ml	Positive for anti-HSV 1&2 IgG

2.3.2 Real Time PCR to quantitatively determine CMV DNA

This protocol was adapted from the Clinical Microbiology Laboratory at the Royal Liverpool University Hospital after permission of Dr Mark Hopkins. Principally, the protocol is based on the assay described by Boeckh and colleagues (Boeckh *et al.*, 2004) where double primer sets were used in conjunction with a Roche Lightcycler 480 Probe Master mix to amplify regions of the UL55 and UL123 CMV genes. FAM labeled Taqman probes was used to detect CMV amplicons in the relevant fluorimeter channel of the Roche LC480 instrument. In addition a second heterologous amplification system was used to identify possible PCR inhibition. Herein, EXO-DNA was used as internal control (IC), which was detected with HEX labeled probe in a different fluorimeter channel. Note in addition to EXO-DNA all the primers and probes were purchased from Eurofins MWG Operon. The standard curve for measuring the CMV virus load was created by using serial dilutions of UL55 and UL123 plasmid clones. An Invitrogen pCRII-TOPO TA cloning kit was used to generate plasmid sizes (including the PCR product) of 4039 and 4059 bases for UL55 and UL123 respectively. See Table 2-5 for probe and primer specifications.

Table 2-5: Primers and probes for Quantitative Detection of Cytomegalovirus DNA in Plasma by Real-Time PCR

Primer set	Region	Amplicon size (bp)	Forward primer	Reverse primer	Probe (Fluorochrome)
CMV	CMV-UL123-exon4	84	TCC CGC TTA TCC TCR GGT ACA	TGA GCC TTT CGA GGA SAT GAA	TCT CAT ACA TGC TCT GCA TAG TTA GCC CAA TAC A (FAM)
CMV	CMV-UL55	68	TGG GCG AGG ACA ACG AA	TGA GGC TGG GAA GCT GAC AT	TGG GCA ACC ACC GCA CTG AGG (FAM)
EXO-DNA	EXODNA	89	GCC TGG TGC AAA AAT TGC TTA	TCG TTC ATT TGT TCT TTT GTG GAA	CAG CTA TTG CAA ACG CCA TCG CAC (HEX)

RT-PCR Procedure

2.3.2.1 Nucleic acid extraction

DNA was harvested from 100µl of blood. A QIAamp DNA Mini Kitkit (Qiagen, product number; 69504) was used and the protocol supplied by the manufacturer was followed. Basically, 100µl of whole blood was placed in a microcentrifuge tube containing 20µl of proteinase K, and 200µl of lysis buffer. The mixture was vortexed and incubated for 10 minutes at 56°C. After the incubation period, 200µl of 98-100% ethanol was added to the mixture and the tube inverted. After that, the mixture was pipetted into a QIAamp Mini spin column placed in a 2ml collection tube and centrifuged for 1 minute at 6000g (8000rpm) then the flow-through and collection tube were discarded.

The mini spin column was placed in a new 2ml collection tube and washed twice with washing buffers (labeled as AW1 and AW2) for 1 minute at 6000g (8000rpm) and for 3 minutes at 20,000g (14000rpm) respectively. The flow-through and collection tube were discarded and the DNeasy mini spin column was placed in a new 2ml collection tube. Finally, 200µl of buffer AE (elution buffer) was added to the mini spin column and incubated for 1 minute at room temperature and then centrifuged for 1 minute at 6000g (8000rpm); this step was repeated to increase the overall of DNA yield, see Figure 2.3

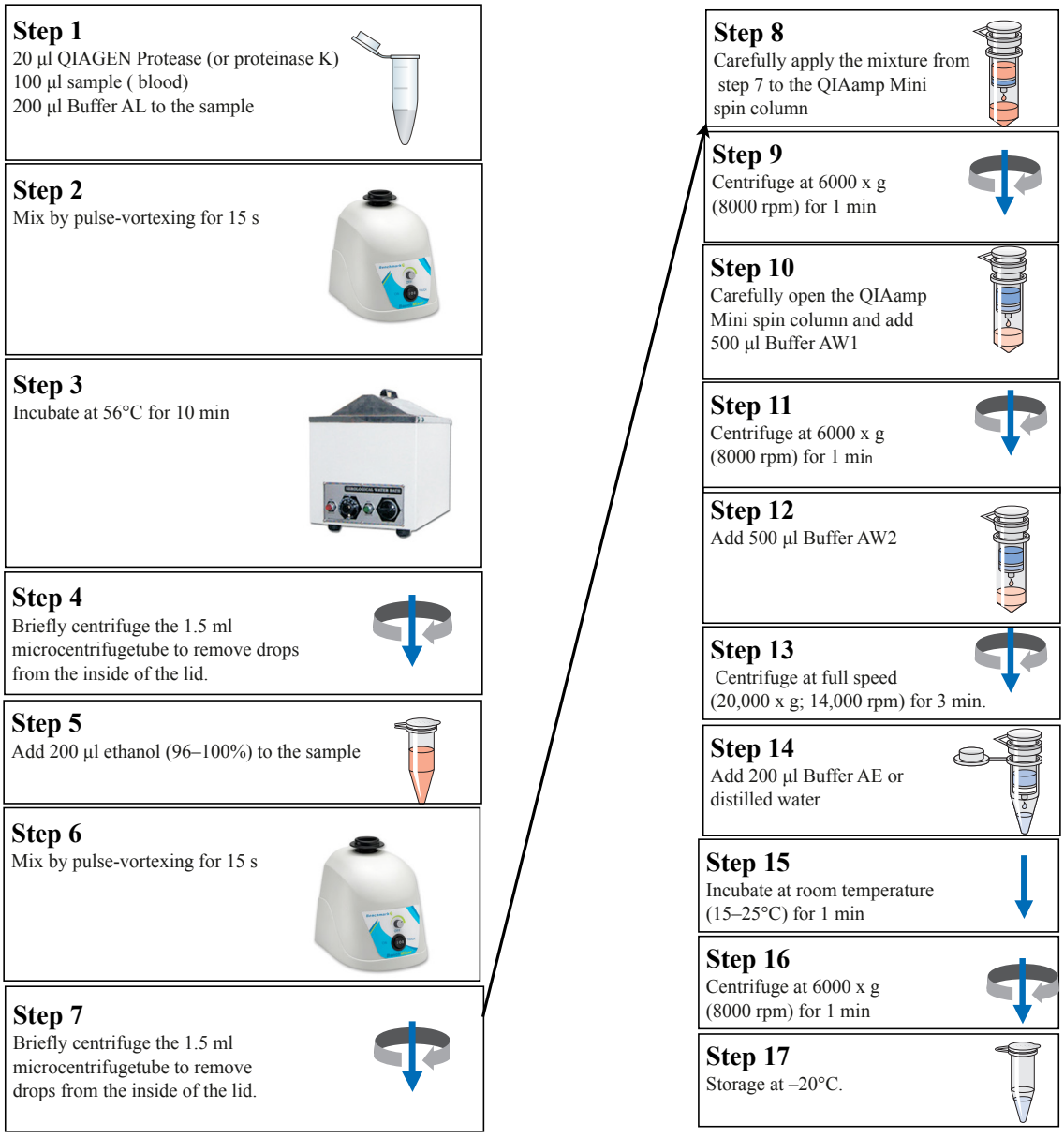


Figure 2.3: Summary of steps for DNA extraction from blood utilizing a QIAamp DNA Mini Kit

2.3.2.2 Preparation of primers and probes

New primers were re-hydrated with nuclease-free water to a stock concentration all at 100µM. Regarding the working solution the CMV primers (UL55F, UL55R, UL123F, and UL123R) were all adjusted to 10µM. Meanwhile, working concentration for the internal control primers (IC) was 5µM for EXO315R and 2.5µM for EXO186F.

New taqman probes were re-hydrated with TE buffer (10 mM Tris-Cl, pH 7.5 and 1 mM EDTA) to stock concentrations of 100µM. The concentration of working aliquots of the CMV probe mix (UL55-FAM and UL125-FAM) was 10µM and for the internal control (EXO-HEX) was 5µM. To avoid repeated freeze/thaw of primers and probes aliquots were prepared and stored in separate tubes at -20C and only mixed during the preparation of master mix, see Figure 2.4.

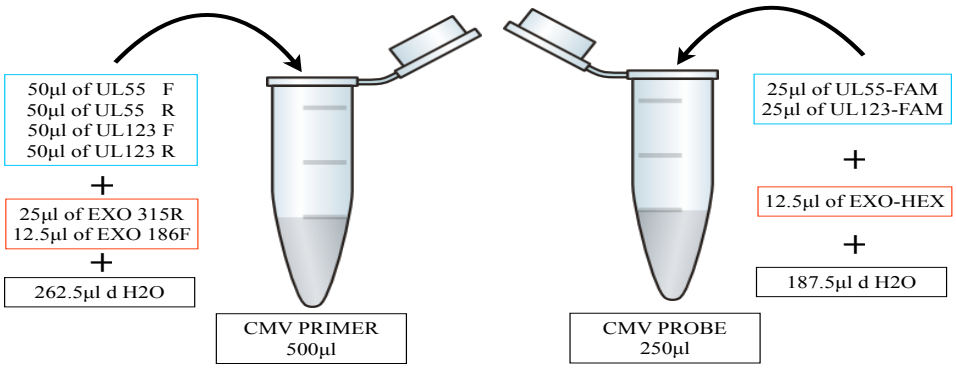


Figure 2.4: Preparation of primer and probe aliquots

The primers for CMV (UL55FUL55R, UL123F, and UL123R) and IC (EXO315R and EXO186F) and their taqman probes (FAM and HEX respectively) were prepared and kept separate until the time of master mix preparation.

2.3.2.3 Master mix preparation

The amount of master mix prepared each time was dependent on the number of samples proposed, see Table 2-6:

Table 2-6: Master mix preparation

Component	1*	2*	4*
Roche probe master	12.5	25	50
CMV primer mix	1	2	4
CMV probe mix	0.25	0.50	1.00
H_2O	1.25	2.50	5.00

*Indicates number of samples.
All volumes are measured in µl
15 µl of this master mix will be added to 10µl of patient purified DNA sample.

Note; to check for PCR inhibition the internal control was included in each well therefore 0.25µl of water was substituted by Exo DNA (10^6 copies/ml) in 15µl of LC480 master mix prior to addition of 10µl purified sample.

2.3.2.4 Amplification setup

An LC480 Roche instrument and software were used to run the sample and set up to read multicolour hydrolysis of probes (FAM and HEX). The protocol that was used is shown in the Table 2-7:

Table 2-7: Amplification protocol

Protocol	Temperature ^{0}C	Time at temp (min:ss)	Ramp rate (C^{o}/sec)	Acquisition mode
Pre-incubation	95	05:0	4.4	None
Amplification 45 cycles of	95	00:10	4.4	None
	60	00:45	2.2	Single
	72	00:10	4.4	None
Cool	40	00:30	2.2	None

2.4 Cellular work

2.4.1 Cell surface staining with monoclonal antibodies (mAb)

For optimal mAb staining in all types of sample used (freshly isolated or cryo-preserved PMBC), the cells-to-media volume ratio was corrected. The ideal concentration for optimal antibody staining was chosen to be 0.5-1x10^6 cells per 30-50µl media. This was based on the recommendation of mAb supplier companies. To obtain this concentration cell pellets were firstly pelleted by centrifugation at 1500rpm for 5 minutes with 4-5ml of FACS buffer. Then the pellet was re-suspended in the appropriate volume of FACS buffer, so that 50µl media will contain at least 0.5×10^6 PBMC. This was followed by incubation of PBMC with fluorochrome-conjugated monoclonal antibodies in ice for 20-30 minutes. Lastly cells were washed by FACS buffer and to prevent the flow cytometer from blocking all samples were filtered through a 70µm Nylon cell strainer (BD Falcon) just before flow cytometric analysis was carried out.

Note, the quantities of monoclonal antibodies were adjusted according to fluorescent brightness, concentration of the antibodies and density of target molecules on the cell surface. Therefore, each antibody was independently titred to give the optimal staining quality before carrying out the actual experiments. Also mouse isotype monoclonal antibodies were usually included with each experiment set.

Since stimulation of PBMC is known to cause alteration in the expression level of some cell surface activation markers such as co-stimulatory molecules, therefore, most phenotyping studies were carried out using unmanipulated cells (not *ex-vivo* stimulated). Panels of monoclonal antibodies including the type of fluorescent dyes (fluorochrome) conjugated, and quantities that are used in each experiment are summarized in Table 2-8.

All incubations of surface staining were on ice and for 25 minutes followed by a washing step with FACS buffer and centrifugation (400g) for 5 minutes. However, staining with tetramer/pentamer was slightly different, see section 2.4.2 for detailed tetramer/pentamer staining and for panels that include an intracellular staining step see section 2.4.3.

Table 2-8: Monoclonal antibody staining panel for lymphocyte phenotype profile

Tube	Purpose	FITC (Green)	PE (Yellow)	PerCP-Cy5.5 (Far red)	APC (Red)
1	**Unstained**	None	None	None	None
2	**FITC control**	1µl /CD8	None	None	None
3	**PE control**	None	1µl /CD8	None	None
4	**PeCy5.5 Control**	None	None	1µl /CD8	None
5	**APC control**	None	None	None	1µl /CD8
6	**αβ T cell profile**	1µl /CD27	1µl / CD8	1µl / CD28	1µl/ CD4
7	**NK cell profile**	2µl / CD16	1µl / NKG2C	1µl / CD3	1µl/ CD56

This table summarizes the surface-staining panel of monoclonal antibodies that are used to identify different lymphocyte phenotypes. Each column in the table was given the colour corresponding to the colour of antibody-conjugated fluorochrome. The volumes (µl) denote the quantity of each antibody used per 50 µl of cell suspension (approx. $0.5x10^6$ PBMC)

Table 2-9: Summary of *γδ* T cells surface and intracellular monoclonal antibody staining panel.

Tube	Purpose	FITC (Green)	PE (Yellow)	PerCP-Cy5.5 (Far red)	APC (Red)
1	Unstained	None	None	None	None
2	FITC control	1µl /CD8	None	None	None
3	PE control	None	1µl /CD8	None	None
4	PeCy5.5 Control	None	None	1µl /CD8	None
5	APC control	None	None	None	1µl /CD8
6	γδ T cell subsets	1µl / TCR Vδ1	1µl / TCR Vδ2	None	1µl/ TCRγδ
7	αβ & γδ T cell profile	3µl /TCR αβ	1µl / TCR Vδ2	None	1µl/ TCRγδ
8	Differentiation status	1µl / CD27	1µl / TCR Vδ2	1 µl / CD28	1µl/ TCRγδ
9	Naive/memory status	1µl / LFA-1	1µl / TCR Vδ2	1µl / CD45RA	1µl/ TCRγδ
10	T cell regulation	2µl / TCR Vδ2	1µl / PD-1	1µl / CD28	1µl/ TCRγδ
11	Proliferative ability	1µl / IL7R	6µl / IL15R	1µl / TCR Vδ2	1µl/ TCRγδ
12	Cytotoxic ability	5µl / Perforin	1µl /Granzyme B	1µl / TCR Vδ2	1µl/ TCRγδ

This table summarizes the design of experiments that used surface staining as well as internal staining of PBMC by monoclonal antibodies. In colour control tubes (tubes from 1 to 4) there was $0.5x10^6$ PBMC incubated with only one specific anti-human monoclonal antibody. Meanwhile in tube 5 to 10 cells were incubated with combinations of different coloured conjugated anti-human monoclonal antibodies.

2.4.2 Tetramer/Pentamer staining of CMV–specific CD8+ T cells

Tetramer/pentamer staining was conducted on Human Leucocyte Antigen (HLA) matched donors. Thus 2 ml blood from CMV positive donors was sent to the tissue-typing unit in the Royal Liverpool Hospital for HLA typing. Based on availability of tetramer/pentamer, only donors with HLA-A*01, HLA-A*02, HLA-B*07, or HLA-B*08 (the most common European alleles) were considered for this analysis. The tetramer/pentamer selected for this purpose was loaded with CMV epitopes known as the most immunodominant, which involves pp65, and IE2 matrix protein of CMV, (Wills *et al.*, 1996b, Kern *et al.*, 2002, Khan *et al.*, 2004).

Note, tetramer was freshly prepared by Dr. Khan, where appropriate monomers of alleles that were loaded with CMV epitopes were conjugated to an APC or PE fluorescently labelled streptavidin molecule via biotin. N.B, the detailed method for tetramer preparation is described elsewhere (Altman *et al.*, 2010).

For tetramer staining the following protocol was followed; PBMC were isolated (as discussed in section 2.1) and cells suspended at approx. 10^6 cells per 100µl growth media. 2 µl of tetramer was added per tube and incubated at 37°C for 20mins, then washed in PBS (650xg for 5min). Cells were then surface stained with CD8 and any other antibodies indicated and/or intracellular stained in accordance with protocols in sections 2.4.1 or 2.4.3 respectively.

Pentamers were purchased from ProImmune under the name of Pro5® MHC Pentamers, see Table 2-10. Besides their stability Pro5 MHC Pentamers are unlabeled therefore there was flexibility in choice of fluorescent label (here called Fluorotag), in this thesis PE and APC- Fluorotags were used. Note that the protocol was similar to the tetramer

staining protocol mentioned above, however, the only difference is to add the appropriate amount of fluorotag during the CD8 surface staining step.

Table 2-10: combination of pentamers allele and CMV-peptide sequence

ALLELE	PEPTIDE SEQUENCE	EPITOPE ORIGIN
A*01:01	VTEHDTLLY	HCMV pp50 245-253
A*02:01	NLVPMVATV	HCMV pp65 495-504
B*07:02	TPRVTGGGAM	HCMV pp65 417-426
B*08:01	QIKVRVDMV	HCMV IE1 88-96

2.4.3 Intracellular cytokine staining (ICS)

It is known that incubation of PBMC with viral lysate (or pool of purified peptides) induces pro-inflammatory cytokine production (Radha *et al.*, 2005). Based on this, frequencies of virus-specific CD4+ T cells and CD8+ T cells were measured by determining the increase in production of intracellular cytokine, which in our study one or all of the following cytokine were targeted; IFN-γ, TNFα and/or IL-2. Steps of intracellular staining are summarized in Figure 2.5.

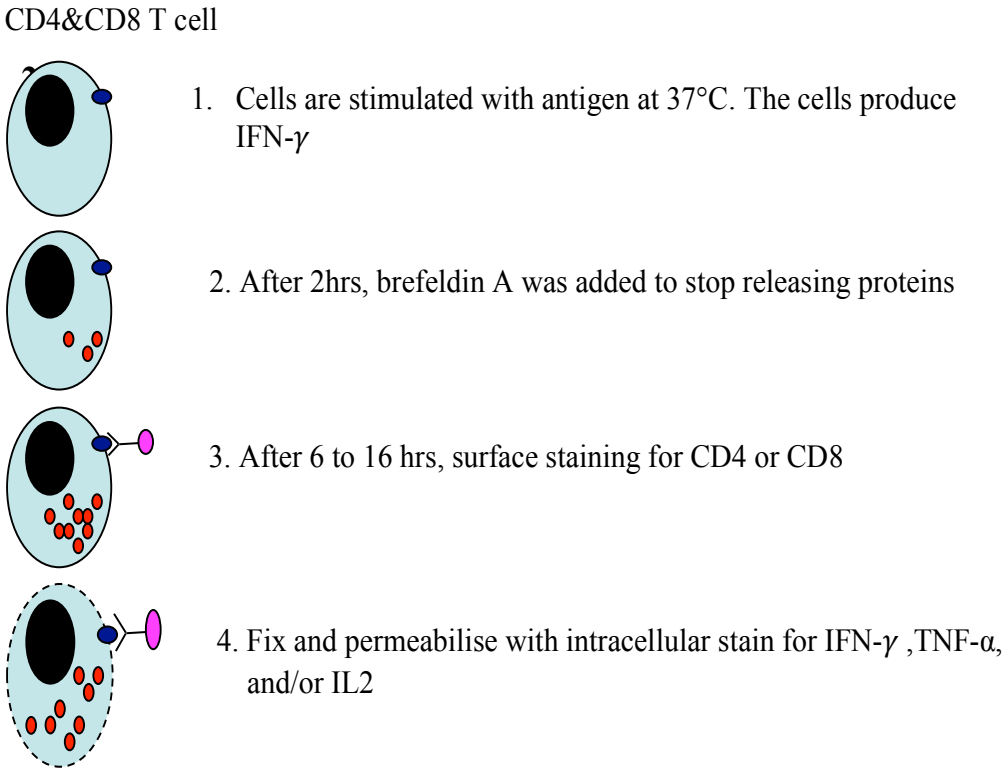


Figure 2.5: Steps for intracellular mAb staining

Showing the action of each step of the stimulation assay. In the first step the cells were stimulated by viral-Ag and this antigen enhances the cells to secret the cytokine such as, IFNγ. After two hours of incubation the secreted molecules were inhibited by Brefeldin A. After 6-18 hours the cells were labeled by fluorochrome–conjugated anti-CD4 and/or anti-CD8. Then the cells were fixed and permeabilized for intracellular staining such as, IFN-γ. Ultimately, the cells were analyzed by flow cytometry.

2.4.3.1 T cell responses to CMV

Aiming to determine CMV-specific CD4+ T cells responses, 0.5-1×10^6 freshly isolated PBMC were incubated with lysate of CMV-infected fibroblast or mock lysate (made from non-infected fibroblasts "negative control") at 37°C for a total of 6-14 hours. Meanwhile, to stimulate CD8+ T cells, cocktails of known immunogenic CMV peptides (made commercially by Invitrogen, UK) were used. The antigens selected were IE1, pp65 and a combination of pp50 and gB peptides (VTE/DYS). Collectively, these antigens have been shown to stimulate responses in the main percentage of previously infected persons. More details about peptide are shown in Table 2.11. At the end of the second hour of incubation, 400µl of Brefeldin A was added at 10µg/ml final concentration. The aim of this step is to prevent the export of cytokine into the extracellular environment, so that it accumulates within the cell and allows detection using intracellular antibody staining later. After incubation for the remaining hours, cells were washed with FACS buffer. Then, they were incubated with monoclonal antibodies (mAb) for surface staining of CD4 and/or CD8.

To fix the surface antibodies and prevent them from being removed during the permeabilization step, samples were washed by FACS buffer, and then incubated with 80µl fixation reagent (intraprep reagent 1; Beckman Coulter, UK) in the dark for 20 minutes at room temperature. To allow entry of antibodies to the cytoplasm, cells were incubated for 5 minutes with 80µl of permeabilizing agent (intraprep reagent 2; Beckman Coulter, UK). Without washing away the permeabilizing reagent, cells were incubated with anti fluorochrome-conjugated anti-cytokine (IFNγ, TNFα, and/or IL-2) mAb for 30min at room temperature, before a final wash.

Table 2-11: Immunogenic CMV Peptides and the class I HLA alleles they are bound by

CMV antigen	Peptide	HLA type
IE1 MIX	VLEETSVML	A2
	CRVLCCYVL	B7
	ELRRKMMYM	B8
	QIKVRVDMV	B8
	KEVNSQLSL	B40
	EEAIWYTL	B44
	FPKTTNGCSQA	B55
	EVISVMKRR	A68
	ATTFLQTMLR	A68
	KRKMIYMCY	B27
pp65 MIX	NLVPMVATV	A2
	TPRVTGGGAM	B7
	RPHERNGFTV	B7
	IPSINVHHY	B35
	FPTKDVAL	B35
	QPSLILVSQY	B35
	YSEHPTFTSQY	A1
	QYDVPAALF	A24
	QVVKVYLESF	A24
	YTPDSTPCHR	A68
	DTPVLPHETR	A68
	PQYSEHPTRTSQYRIQ	DR11
	FTSQYRIQGKLEYRHT	DR11
	KYQEFFVDANDIYRI	DR52
	IIKPGKISHIMLDVA	DR4/DR53
	AGILARNLVPMVATV	DR3/DR11
VTE/DYS MIX	VTEHDTLLY	A1
	DYSNTHSTRYV	DR7

2.4.3.2 Quality of CMV-specific T cell responses in patients with coronary artery disease (CAD)

Referring to chapter 5, the aim was to determine differences in the magnitude of T cell responses to CMV in patients with coronary artery disease and age-matched healthy volunteers. A BD LSR II flow cytometer was utilized to define the frequency of CMV-specific T cells that are capable of exhibiting multifunctional properties. Here a slightly modified intracellular staining protocol was used. Briefly, PBMC from CAD patient and healthy donors were stimulated with a mixture of IE-1 and pp65 CMV peptides and CMV-fibroblast lysate for approximately 10 hours in the presence of co-stimulatory antibodies (purified anti-CD28 and anti-CD49d mAb, both from BD), Golgi Stop containing monensin besides brefeldin-A (BFA), and FITC conjugated anti CD107a (as a degranulation marker) monoclonal antibody. On day two, surface staining for CD4-PerCP-Cy5.5 and CD8-APC/Cy7 was carried out followed by intracellular staining with mAb detecting four main functions including; anti MIP1β-PE, IFNγ-AlexaFluor700, TNFα-PE/Cy7, and IL-2-APC. Note, all of these mAb were purchased from BD Biosciences.

2.4.3.3 T Cell responses to VZV antigen

The aim here was to measure the frequency of VZV–specific T cells. Therefore, similar to the CMV work, ICS assays were used to detect the increase in inflammatory cytokine production by T cells in response to VZV antigen. The same protocol as in section 2.1.5.1 was used and the only difference was in the stimuli which were either 1µl (concentration 0.72mg/ml) of VZV Rod strain inactivated cell extract (Advanced Biotechnologies Inc) or 10µl (concentration $10^{3.3}$ PFU/0.5ml) live attenuated VZV-varilrix vaccine (Glaxo SmithKline).

2.4.4 CFSE assay

CFSE (Carboxy-Fluorescein Succinimidyl Ester) proliferation assay was utilized to identify the ability of T cells to proliferate in response to VZV antigen. Briefly, this assay is based on the ability of CFSE molecules to diffuse into cells. Therefore, when CFSE is incubated with live cells, endogenous enzymes such as, esterases process it and it tends to bind to the cytoplasmic membrane and also to nuclear proteins. Upon entering the cell the CFSE diffuses uniformly throughout the cytoplasm, therefore when the cells divide the CFSE dye is split equally between the daughter cells. Consequently the CFSE concentration in each cell will reduce as the cells proliferation progress. Using flow cytometry the amount of fluorescence emitted by each generation of cells will be inversely proportional to the number of divisions.

2.4.4.1 Labelling of cells with CFSE

PBMC were isolated in the usual way, followed by cell counting of viable cells and adjusting the concentration so that each ml of PBMC suspension contained 2 to 3 million cells. CellTrace^{TM} CFSE Cell Proliferation Kit *(*Invitrogen) was used to perform the CFSE labeling.

Briefly, a stock solution of 5mM CFSE was prepared by adding 18µl of DMSO (Component B) to one vial of CFSE (Component A)**.** A titrated amount of CFSE solution (2.5-5µl) was then added to PBMC, mixed gently and incubated for 10min at 37°C. This was followed by three washing steps, each time using 10ml of cold PBS, and centrifugation for 10 minutes at (400g) was used. Finally recounting of the viable cells was performed and cells were re-suspended in growth media at a concentration of 1-1.5x10^6 per ml per well.

2.4.4.2 Cell stimulation

Experiments were designed so that in addition to the VZV-antigen (1µl of purified VZV peptides or 15µl of vaccine) stimulated wells for each donor there were duplicates of positive control, Phytohaemagglutinin (PHA 0.5µl), and negative control (no stimulus) wells**.** Eventually, the plate was incubated for 7 days at 37°C.

2.4.4.3 Staining of cells

On day 8, the plate was removed from the incubator and the desired number of PBMC was aliquoted from the cell suspension into labeled FACS tubes. This was followed by washing steps, which included adding 4ml of FACS buffer to each tube and spinning at 1500rpm for 5min in the centrifuge. Next, the supernatant was discarded and cells resuspended in the residual volume of buffer. Next, antibodies for surface staining to both test samples and control samples were added and incubated on ice for 20min. The staining panel involved anti-CD4/PE and anti-CD8/APC mAbs. This was followed by washing off unbound antibodies with 4ml of FACS buffer and spinning at 1500rpm for 5min in the centrifuge.

Finally, cells were resuspended in 400µl of 'FACS fix' solution (PBS containing 2% paraformaldehyde and 2% FCS) and mixed thoroughly by vortexing for 10-15 seconds. Flow cytometric analysis was done using a FACSCalibur flow cytometer where CFSE is detected by FL1 and CD4 and CD8 detected by FL2 and FL4 respectively.

2.5 Ex vivo Cell Culture

2.5.1 Isolation of γδ T cell from peripheral blood

Here the manufacturer's instructions for use of the TCRγδ+ T Cell Isolation Kit (Miltenyi Biotech) were followed. Basically, untouched TCRγδ+cells (γδ T cells) were isolated by depletion of non-TCRγδ cells (non-γδ T cells). Non-TCRγδ cells, which include αβ T cells, NK cells, B cells, dendritic cells, granulocytes, monocytes, stem cells, and erythroid cells, were indirectly magnetically labeled with a cocktail of biotin-conjugated monoclonal antibodies, as primary labeling reagent, and anti-biotin monoclonal antibodies conjugated to Micro-Beads, as secondary labeling reagent. The magnetically labeled non-γδ T cells were depleted by retaining them on a MACS Column in the magnetic field of a MACS Separator, while the unlabeled γδ T cells passed through the column. The efficiency of isolation process was calculated by measuring the frequency of γδ T cells ***pre*** and ***post*** selection by flow cytometry. The aim was to reach 95-100% purity.

2.5.2 Establishment of γδ T cell lines

After the frequency of γδ T cells had been enriched (as described in above) to more than 95 % they was co-cultured with irradiated PHA-activated allogeneic PBMC as feeder cells and stimulated with anti-CD3 and recombinant interleukin-2 (rIL-2). Cultures were enriched further by depletion of αβ T cells prior to re-stimulation with feeder cells (after 3 to 4 weeks) followed by maintenance with media containing rIL-2. The absence of αβ T cells was confirmed by flow cytometry. As controls CMV-specific CD8+ T cell lines were generated from CMV-seropositive donors after stimulation with the cocktail of CMV-encoded peptide epitopes.

2.5.3 Culturing / subculturing (passaging) of Fibroblasts

Human fetal foreskin fibroblasts (HFFF) were all maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 2 mM glutamine, 100 IU of penicillin/ml, 100 µg of streptomycin/ml, and 10% foetal calf serum (FCS). In addition, 1% Amphotericin B was also used to prevent fungi from contaminating cell cultures. The passage of HFFF was carried out whenever >90% of the flask surface became covered by fibroblasts. Briefly, after discarding the old media from the flask it was washed with PBS followed by addition of trypsin-EDTA solution and incubated in 37°C for 2-5 minutes, rocking the flask every minute. When all fibroblasts detached the contents of the flask were transferred into a 50 ml tube where they were washed again with PBS and centrifuged. Finally fibroblasts were resuspended and counted and an appropriate volume of growth media was added according to cell count and the size of flasks.

2.5.4 Infection of HFFF with human CMV

A number of HFFF flasks were prepared as described in section 2.5.3. When the HFFF were at 70% confluence they could be used for infection by CMV. After the maintenance media had been removed, CMV was added to each flask at the appropriate multiplicity of infection (MOI). The amount of virus was dependent on the proposed experiment, see Table 2-12.

Table 2-12: Multiplicity of Infection (MOI)

Purpose	Multiplicity of infection (MOI)Virus: Fibroblast	Number of cells	Amount of virus particles needed
To propagate CMV for production of more viruses	**0.1:1**	**For 10 flasks (size 150cm^2) ($3x10^6$ cells each)**	**$3x10^6$**
To infect of HFFF for preparation of CMV lysate for inducing T cell responses	**4:1**	**For 10 flasks (size 150cm^2) ($3x10^6$ cells each)**	**$12x10^7$**
For infection of targets for use in T cell recognition assays	**Up to 10:1**	**Depends on number ofassays**	**Depends on number of assays**

In this thesis both AD169 and Towne strains of human CMV were used for infection of fibroblasts. The virus and fibroblasts were incubated at 37°C for 90min in 5ml of media, the flasks were rocked every 15-20min. For mock-infected flasks media alone was added. This was followed by washing off of virus with media and incubating in 25ml of media at 37°C. The monolayer integrity of adherent fibroblasts was checked daily for appearance of the cytopathic effect (CPE). Supernatant collection was started upon first signs of CPE in sterile tubes and stored at -80°C. Fresh media was added and cells returned to the 37°C incubator. This step was repeated every 3-4 days until cells showed extreme CPE. When HFFF showed near complete death at this stage they were removed by sterile scraper and transferred into a sterile tube. These cells were used for preparation of CMV lysate that can be used in intracellular cytokine assays. See section 2.5.6.

2.5.5 Preparation of CMV

Aiming for propagation of CMV virions, the growing HFFF were infected at multiplicity of infection (MOI) of 0.1:1**.** All the supernatants collected during the culture period were

initially centrifuged at 1500 rpm for 10min to remove any cell debris. The cleared supernatants were then pooled into large centrifuge pots. This was followed by centrifugation at high speed (12000 rpm using a GSA Sorvall) for 2 hours. Next supernatant was decanted and virus pellet was resuspended in about 5ml of RPMI. Finally, a 21G needle was used to aspirate the cloudy fluid and aliquot into sterile eppendorf tubes at 50-100µl per tube and transferred into a -80°C freezer.

2.5.6 Fibroblast lysate

Fibroblast monolayers were grown as described in section 2.5.3. We usually aimed to have ten 150cm^2 flasks ready for infection and five flasks for mock-infected controls. Infection of fibroblasts with AD169 or Towne strains of human CMV was carried out at MOI of 4:1 as explained in section 2.5.4. The monolayer integrity of adherent fibroblasts was checked daily for appearance of the cytopathic effect, which was usually visible within 16 hours (overnight). The first harvesting of supernatant from each flask was done on day 8 and frozen at -80°C. More media was added to cells and incubation at 37°C was continued. Harvesting of the supernatant then continued twice weekly from then on. When most of the monolayer became detached from the plastic, cells were harvested, using a scraper if necessary. Cultures normally survived for 2-3 weeks. Next cells and debris were separated and the pellet retained from the supernatant by spinning in a separate container. To make lysate the pellets and cell debris were snap frozen in liquid nitrogen and reheated to 50°C several times. For safety reasons the CMV lysate was exposed to UV light to ascertain that any remaining virus was totally inactivated.

2.6 Flow cytometry

Most analyses were carried out on a BD FACSCalibur flow cytometer (BD Biosciences). Multiple parameters were used to define the lymphocytes, including gating, based on forward scatter (FSC) and side scatter (SSC). Fluorescence emission was measured at different wavelengths: FL1: 519 nm (FITC), FL2: 578 nm (PE/ Cy3), FL3: 695 nm (PerCP-Cy5.5), and FL4: 660 nm (APC).

Unstained cells were used to set the background levels of fluorescence so that <1% of cells were determined as positive for any of the colours used. Cells incubated with single colours; CD8-FITC, CD8-PE, CD8- PerCP-Cy5.5, and CD8-APC were used to verify detection of each individual colour by the flow cytometer. Colour compensation was carried out when false positive results were obtained. For example, if FL4 (APC) positive events were also observed in samples only stained with PC5.5-conjugated anti-CD8, the flow cytometer settings were corrected – i.e. compensated to show events as FL4 (APC) negative. This compensation was applied for each colour in order to avoid false positive results in test samples, where antibodies with different colours were used.

A BD LSR II flow cytometer was also accessible to perform some studies in chapter five (role of CMV in coronary heart disease). In this project the following fluorochrome-mAbs were used; for surface staining CD107-FITC, CD4-PerCP-Cy5.5 and CD8-APC/Cy7 while for intracellular staining anti MIP1β-PE, IFNγ-AlexaFluor700, TNFα-PE/Cy7, and IL-2-APC monoclonal antibodies were used. In addition, Tracking and Compensation beads (BD Biosciences) were used for voltage setup and auto-compensation process respectively.

CellQuest software and FACSDiva software (both from BD) were used for flow cytometer data acquisition by the FACSCalibur and LSR II, respectively. However, all post acquisition analysis was carried by FlowJo software (version 9.5.2 and X ; Tree Star, Inc).

2.7 Statistical analysis

Since the data from this project did not always follow a Gaussian distribution, unless otherwise mentioned, nonparametric tests were used for all analysis. The median with 25^{th} and 75^{th} percentiles was used for the description of distributions. Meanwhile, for comparison purposes (P value) either the Mann Whitney test (in case of comparing two independent variables) or the Kruskal-Wallis test followed by Dunn's post-test (more than two variables compared) were used. In addition, for association and/or correlation the Spearman test was also used. All P values were two-tailed and 95% significance was applied.

Note all statistical analyses were performed with Graphpad Prism software (version 5-mac, GraphPad Software Inc.). However, for appropriate analysis of polychromatic flow cytometry data (Chapter 5) special programs called PESTLE and SPICE v5 (Simplified Presentation of Incredibly Complex Evaluations, Version 5) (Roederer, 2011) were used to generate graphs and for statistical analysis.

N.B, More description of statistical testes will be included in separate section with each chapter.

2.8 List of reagents and antibodies

2.8.1 Reagents

1 Ficoll Hypaque density gradient centrifugation (GE Healthcare, product number; 17-1440-03).
2 Phosphate-buffered isotonic saline (PBS) (Sigma, product number; P4417)
3 RPMI 1640 medium (Sigma, product number; R0883)
4 Dulbecco's modified Eagle's Medium (DMEM) (Sigma, product number; D5796)
5 Fetal bovine serum (FBS), (Sigma, product number; F4135).
6 Glutamine (2mM), penicillin (50u/ml), and streptomycin (Sigma, product number; G6784)
7 Brefeldin A (Sigma, product number; B7651)
8 Phytohemagglutinin-PHA-M (Sigma; product number: L8902)
9 Bovine serum albumen (BSA) (Sigma, product number; A2153)
10 EDTA (BDH, product number; 104245S)
11 Ethanol (University of Liverpool)
12 Distilled water DNAse and RNAse free (Gibco, product number; 10977-035)
13 Leucocytic Permeabilization Reagent (IntraPrep ; Beckman Coulter product number:A07803)

Reagent 1 (Fixation agent) formaldehyde.

Reagent 2 (permeability agent) saponin.

2.8.2 Monoclonal antibodies

Table 2-13: Antibodies used in multiparameter T cell studies (chapter 5)

Target	Fluorochrome	Clone	Isotype	Concentration	Catal -Number	Vendor
IFN-γ	Alexa Fluor 700	4S.B3Mouse	IgG1	1/50	502520	BioLegend
TNF-α	PE-Cy7	MAb11	IgG1	1/50	557647	BD Biosciences
IL-2	APC	5344111	IgG1k	1/50	341116	BD Biosciences
MIP-1β	PE	D21-1351	IgG1	1/50	550078	BD Biosciences
CD4	eFluor 450	RPA-T4	IgG1k	2/50	48-0049-42	eBioscience
CD8	PerCP-Cy5.	5RPA-T8	IgG1k	1.5/50	560662	BD Biosciences
CD107a	FITC	H4A3	IgG1k	5/50	555800	BD Biosciences

Table 2-14: Monoclonal antibodies used in NK cell phenotype studies

Target	Fluorochrome	Clone	Isotype	Concentration	Cata-Number	Vendor
CD16	FITC	NKP15	IgG1k	1/50	347523	BD Biosciences
CD3	PerCP-Cy5.5	OKT3	IgG1	1/50	45-0037-73	eBioscience
CD56	APC	MEM188	IgG1	1/50	17-064-42	eBioscience
NKG2C	PE		IgG1	1/50	FAB138P	R&D systems

Table 2-15: Monoclonal antibodies used in γδ T cell studies (chapter 4)

Target	Fluorochrome	Clone	Isotype	Concentration	Catalogue - Number	Vendor
TCR γδ	APC	B1	IgG1k	1/50	561049	BD Biosciences
TCR Vδ2	FITC	B6	IgG1	1/50	562088	BD Biosciences
TCR Vδ2	PE	B6	IgG1	1/50	555739	BD Biosciences
TCR Vδ2	PerCP	B6	IgG1k	1/50	331410	BioLegend
TCR Vδ1	FITC	TS8.2	IgG1	1/50	TCR2730	Perbio
TCR αβ	FITC	WT31	IgG1	1/50	347773	BD Biosciences
CD11a (LFA-1)	FITC	G43-25B	IgG2a	1/50	555379	BD Biosciences
CD45RA	PerCP-Cy5.5	HI100	IgG2b	1/50	304122	BioLegend
CD27	FITC	M-T271	IgG1k	1/50	555440	BD Biosciences
CD28	PerCP-Cy5.5	CD28.2	IgG1k	1/50	302922	BioLegend
IL-7Ra	FITC	eBioRDR5	IgG1k	1/50	11-1278-42	eBioscience
IL-15Ra	PE	JM7A4	IgG1k	1/50	330207	BioLegend
Granzyme B	FITC	GB11	IgG1 k	2/50	560211	BD Biosciences
Perforin	PE	dG9k	IgG2b	2.5/50	308105	BioLegend

3 Imprint Of Chronic CMV Infection On The Immune System Of Healthy Subjects

3.1 Background

Primary CMV infection is usually asymptomatic and takes place during childhood. This is followed by persistent lifelong latent infection (Gandhi and Khanna, 2004). Although the exact mechanisms involved in CMV latency are not fully understood, it is clear that different arms of the immune system are involved in keeping viral replication under check (Polic *et al.*, 1998). Being one of the largest known viruses, with a genome of 235 kb, CMV elicits a very broad spectrum of immune responses. It starts with the innate mechanism that includes production of inflammatory cytokines by virus-infected cells and activation of NK cells which ultimately drives the adaptive immune system to produce CMV-specific antibody and generation of αβ T cells and γδ T cell responses (Jackson *et al.*, 2011). The evidence that supports the importance of these different arms of the immune system is discussed in more details in section (1.2.6). However, here more focus was given to certain cell subsets, which are believed to play a direct role in CMV immunity.

Imprint of CMV carriage on the immune system

It has become more evident that chronic CMV carriage has a major influence on the immune system (Gratama *et al.*, 1987). One of the obvious influences might be the effect of CMV on the αβ T cell repertoire. This was particularly true for CD8+ T cells characterised by expression of CD57; this cell subset were found to be monoclonal and correlated positively with human CMV carrier status in healthy individuals (Wang *et al.*, 1993, Wang and Borysiewicz, 1995, Wang *et al.*, 1995). Furthermore, the CD8+ subset shows a double increase in its absolute number in CMV-seropositive elderly compared to CMV-seronegative subjects (Looney *et al.*, 1999). Interestingly, this expansion of

CMV-specific CD8+ T cells can often be monoclonal (Khan *et al.*, 2002b) and characterized by accumulation of highly (late) differentiated effectors memory cells ($CD28^{neg}$ CD8+ T cells) that are can be dysfunctional in the very elderly (Ouyang *et al.*, 2004). Similarly, but at lower frequency, there is an association between an increase in $CD28^{neg}$ CD4+ T cells and CMV seropositivity with advancing age (Pourgheysari *et al.*, 2007). It is important to mention that this phenotype, particularly $CD28^{neg}$ CD8+ T cells, is part of the immune-risk phenotype. In addition, the increase in CD8+ T cell frequency is associated with mortality in the elderly (80-90 years) and considered as a signature of immunosenescence (Olsson *et al.*, 2000, Wikby *et al.*, 2002, Pawelec *et al.*, 2010).

Evidence for the role of NK cells in immunity to CMV in humans comes from several clinical observations. For example patients with a rare NK cell disorder show frequent episodes of acute CMV infection (Biron *et al.*, 1989, Gazit *et al.*, 2004). In addition, recovery of NK cells post bone marrow transplant is associated with a remarkable decrease in incidence of serious CMV disease (Quinnan *et al.*, 1982). Furthermore, encoding of several mechanisms by CMV to evade NK cells is indirect evidence suggesting the importance of NK cells in controlling CMV infection (Wilkinson *et al.*, 2008). Of particular importance, CMV was found to remarkably modify the NK repertoire, for instance, in healthy donors there is a significant increase in frequency of $NKG2C^{+}$ NK cells with reductions in cells with other activating receptors (Guma *et al.*, 2004, Guma *et al.*, 2006, Monsivais-Urenda *et al.*, 2010). In addition, NK cells that were co-cultured *ex-vivo* with CMV infected fibroblasts showed an increase in the expression of NKG2C (Guma *et al.*, 2006). Indeed, such observations highly suggest the importance of $NKG2C^{+}$ NK cells in the control of CMV chronic infection.

Besides NK cells and αβ T cells, the role of non-conventional T cells expressing the gamma delta (γδ) TCR in control of CMV infection has become more appreciated (Lafarge *et al.*, 2001, Knight *et al.*, 2010, Vermijlen *et al.*, 2010).

Unlike αβ T cells, γδ T cells are MHC-unrestricted in their antigen-recognition and normally constitute a minority of circulating T cells. According to the TCR gene usage the γδ T cells can be sub-classified into $V\delta2^{pos}$ γδ T cells and $V\delta2^{neg}$ γδ T cells (Xiong and Raulet, 2007). However, $V\delta2^{neg}$ γδ T cells seem to be more important in CMV immunology. More details about characteristics of this cell subset are reviewed in section (1.2.2.3).

A Pioneering work by Dechanet and colleagues has shown that γδ T cells considerably expand in peripheral blood following primary CMV infection in CMV-seronegative transplant recipients of kidneys from seropositive donor (Dechanet *et al.*, 1999a, Dechanet *et al.*, 1999b). It is worth mentioning that the expansions were composed of either $V\delta1^{pos}$ or $V\delta3^{pos}$ cells but not $V\delta2^{pos}$ cells (Pitard *et al.*, 2008). Furthermore, *in vitro* incubation of $V\delta2^{neg}$ γδ T cells with CMV infected target cells showed the ability of $V\delta2^{neg}$ γδ T cells to produce cytokines and to demonstrate cytotoxicity (Halary *et al.*, 2005) Indeed, such observations highly suggest the importance of $V\delta2^{neg}$ γδ T cells in CMV infection.

3.1 Aims

The influence of CMV carriage on different arms of the immune system has been studied widely. Almost all previous studies focused on the relationship between CMV infection and one kind of cell subset independent of other cell subsets. Therefore, the main aim of the current study is to investigate the simultaneous impact of chronic infection with CMV on different cell subsets in the same healthy donor. Here, the frequencies of the following subsets: $CD28^{neg}$ CD4+ T cells, $CD28^{neg}$ $CD8^{+}$ T cells, $NKG2C^{pos}$ NK cells and $V\delta2^{neg}$ γδ T cells was compared in CMV-seropositive and CMV-seronegative donors.

Since it is not known whether these cell subsets work independently or together to control CMV infection, the second aim was to investigate the existence of association (such as cooperation or redundancy) between these subsets. This was examined by calculating the correlation and linear regression between levels of these cell subsets in the CMV-seropositive cohort.

3.2 Plan of Investigation

To achieve these aims, healthy volunteers from the University of Liverpool and the Royal Liverpool University Hospital (RLUH) NHS Trust were recruited. All subjects were mobile, did not have any cognitive impairment, were not suffering from acute or chronic illness, and were not on medication known to affect the immune system. All blood samples were obtained after formal consent from the donor to participate in the study. Ethical approval for this work had been given by the Liverpool Research Ethics committee. The main steps of the plan of investigation are summarized in Figure 3.1; generally it includes three main steps:

- Determining the CMV status of the cohort.
  - Detecting CMV specific IgG antibody (titre and avidity) by ELISA.
  - Measuring αβ T cell responses against CMV utilizing intracellular cytokine staining (ICS) and flow cytometry.
- Phenotyping to determine the frequency of $CD28^{neg}$ CD4+ T cells, $CD28^{neg}$ CD8+ T cells, $NKG2C^{+}$ NK cells and $V\delta2^{neg}$ γδ T cells in CMV-seropositive donors and compare it to CMV-seronegative donors.
- Analyzing the linear regression of all possible combinations of these cell subsets and calculating the correlation between them.

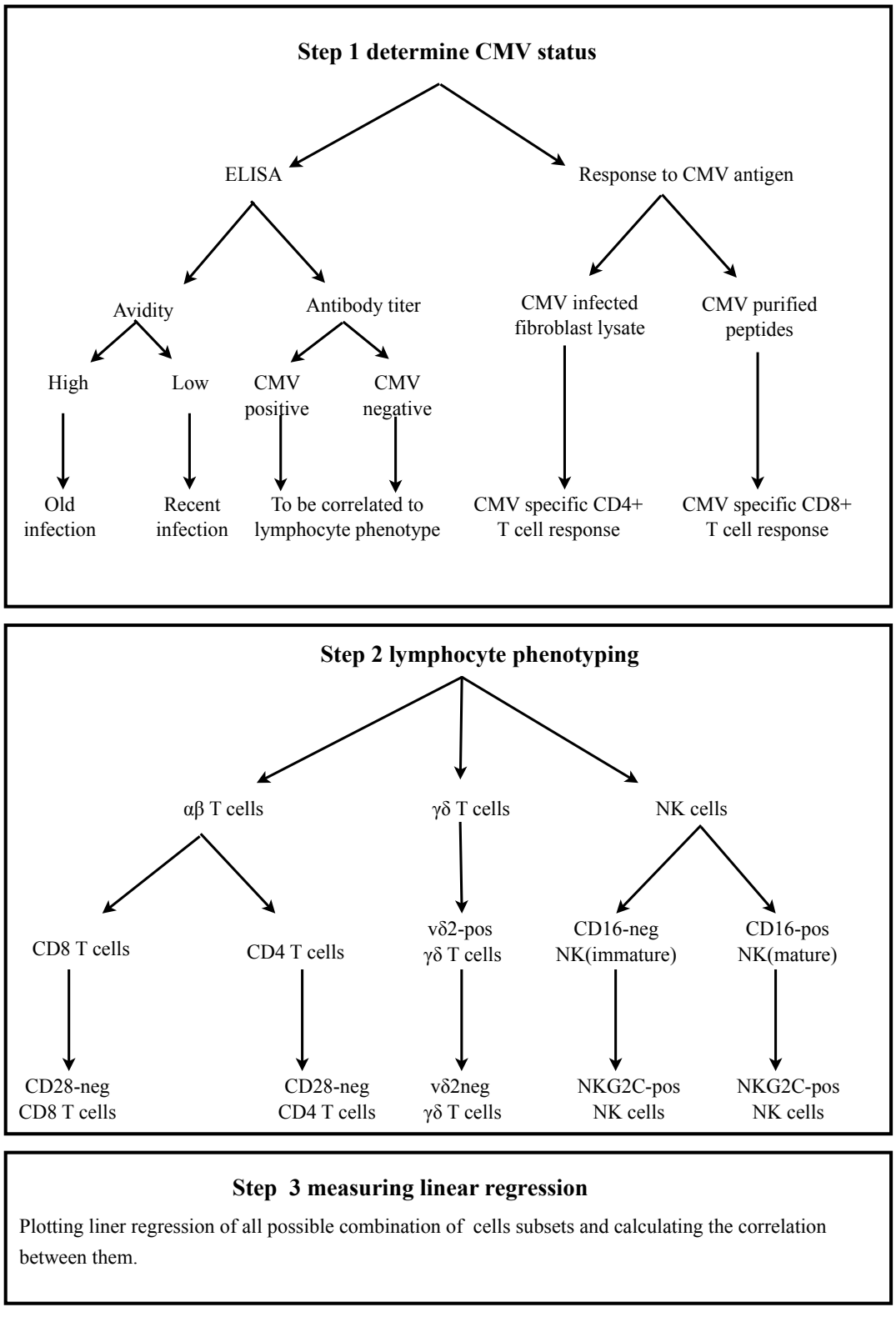


Figure 3.1: Plan for investigation steps.

3.3 Statistical analysis

Since data do not follow the normal (Gaussian) distribution precisely, thus non-parametric tests were used for all analyses. This includes the use of medians with 25th and 75th percentile for distribution description. Meanwhile; Mann-Whitney and Spearman tests were used to calculate the difference in distribution (P value) and the correlation, respectively.

3.4 Results

3.4.1 Testing blood donors for CMV carriage

3.4.1.1 ELISA

To address the aims, it was necessary to confirm the CMV status of the study cohort. This involved the use of two complementary methods to measure humoral and cellular immunity; IgG antibody responses against CMV measured by ELISA, and αβ T cell responses against CMV measured by flow cytometry. 75 consenting volunteers were recruited to donate 10-20 ml of blood. After separation of heparinised blood using Ficoll, plasma was used for quantitative anti-CMV IgG ELISA as described in 2.3.1.1. The ELISA results are summarised in Figure 3.2. According to this assay, out of 75 donors tested there were 36 CMV-seropositive donors.

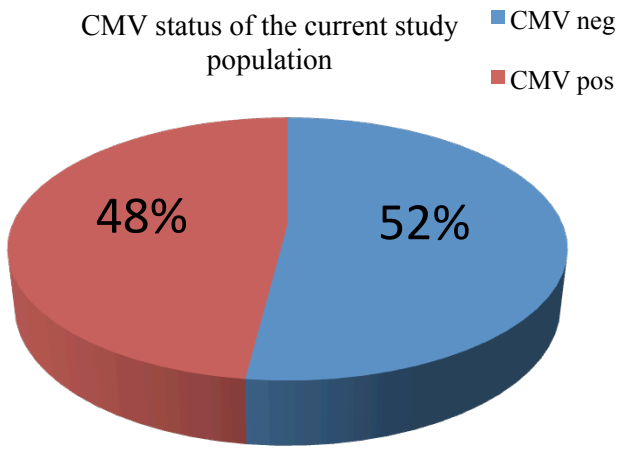


Figure 3.2: CMV status of the study population

This illustrates percentage of CMV seropositive (red colour) and CMV seronegative (blue colour) healthy subjects in current study. The total population of the study is 75 (n=75), 36 of them were CMV-seropositive, while slightly more than the half of donors were CMV-seronegative.

3.4.1.2 Intracellular cytokine staining (ICS)

Simultaneously T cell responses against CMV antigen were carried out. Lysed CMV infected fibroblasts (for more details of CMV lysate see section 2.5.6) was used to measure CD4+ T cells. Meanwhile; cocktails of known immunogenic CMV peptides (made commercially by GPT) were used to measure CD8+ T cell responses. The antigens selected were IE1, pp65 and a combination of pp50 and gB peptides (VTE/DYS). T cell responses were measured by staining PBMC with antibodies against T cell markers (CD4 and CD8) and then against intracellular Interferon-gamma (IFNγ). Figure 3.3 shows three representative examples of donors tested for T cell responses against CMV antigen. The increase in proportions of IFNγ producing CD4+ or CD8+ T cells was used to decide whether there was response or not, the following formula was used to calculate this proportion.

Frequency of CMV-specific CD4+ or CD8+ T cells =

% $IFN\gamma^{pos}$ (CD4+ or CD8+) T cells events in response to mock.

Subtracted from

% $IFN\gamma^{pos}$ (CD4+ or CD8+) T cells events in response to CMV-antigen

In each case there were extremely low responses against mock antigen (0.2-0.5% of CD4+ T cells). However some donors appeared to respond against CMV antigen whereas others did not. In donors HD035 and HD195, 4.5% and 2.28% of total CD4+ T cells were CMV-specific (produced IFNγ) respectively. In other words, proportions of IFNγ production by CMV-specific CD4+ T cell have increased by approximately 90 and 70 times respectively in response to lysed CMV infected fibroblasts. In contrast, in donor HD180 the CMV-specific T cells were at background levels (0.03%) with less than double increases in IFNγ production after stimulation.

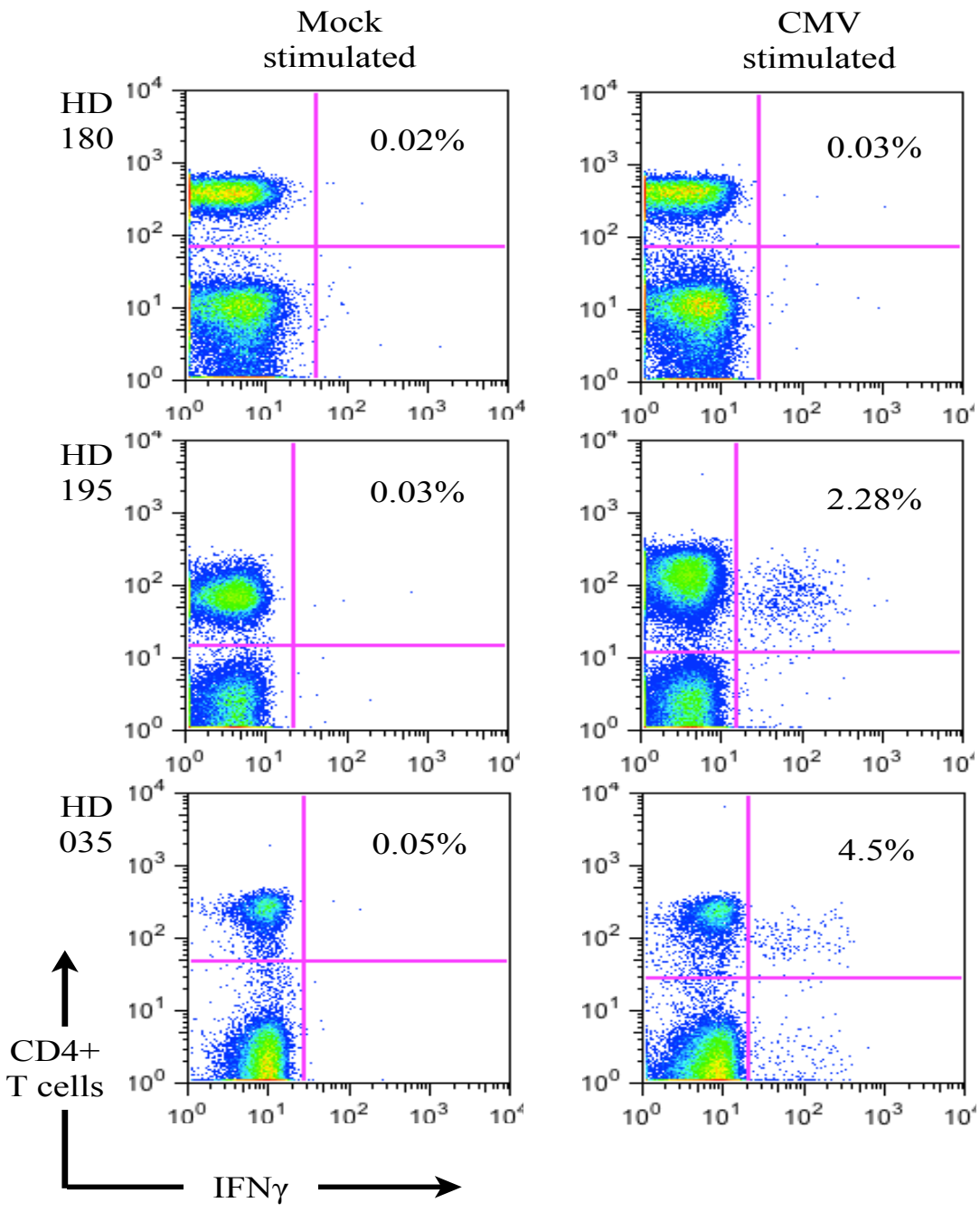


Figure 3.3: Representative flow cytometry plots of healthy blood donor responses against CMV antigens.

This figure shows 3 representative results of blood donor responses against CMV. PBMC were stimulated for 6 hours at 37°C followed by surface staining for CD4-FITC and then intracellular staining for IFNγ-PE. The frequencies shown in the upper right quadrant refer to the % of CD4+ T cells that produce IFNγ out of total CD4+ T cells after stimulation.

3.4.1.3 Measuring CD8+ T cell responses against CMV antigens in CMV carriers

Up to this point, information was only available on CD4+ T cell responses against CMV. Indeed, measuring the CD8+ T cell response to CMV is required to confirm T cell reactivity. However, due to the inefficiency of CD8+ T cell cross priming by exogenous antigens, the CMV antigen (lysate) method is sub-optimal for stimulating CD8+ T cells (Sester *et al.*, 2002a). Instead, an inclusive CMV peptide pools was used for this purpose. These pools spanned the entire amino acid sequences of the HCMV pp65, major immediate early (IE-1) proteins and DYS/VTE. They comprised of 15-amino acid peptides with at least nine overlaps between neighboring peptides. Besides, these reagents do not require further processing and can be presented efficiently to CD8+ T cells. Furthermore, all potential CD8+ T cell epitopes contained in these proteins were provided by the complete pools and, therefore, unlike with single epitopes, testing was somewhat independent of donor HLA type (Kern *et al.*, 2000). Similar to the steps for measuring CD4+ T cell responses, production of IFNγ was exploited as an indicator of cellular responses. Representative examples are shown in Figure 3.4. Notably, from those donors who showed response to CMV lysate/peptide mix, only 10 donors were chosen for this purpose. Briefly, for each donor PBMC were subdivided into three equal amounts and the concentration was adjusted to $1x10^6$ per ml. Then each well was stimulated with a different pool of peptides namely; pp65 peptides, IE-1 peptides and DYS/VTE peptides. Results are shown in Figure 3.5, where the main response was by CD8+ T cells responding to pp65 (mean= 1.68% of total CD8 subset with Standard Deviation (SD) = 1.82) followed by response to IE-1 peptides (mean= 0.99% of total CD8 subset with SD=1.3) and then DYS/VTE mix (mean= 0.93% with SD=0.59), see figure 3.5.

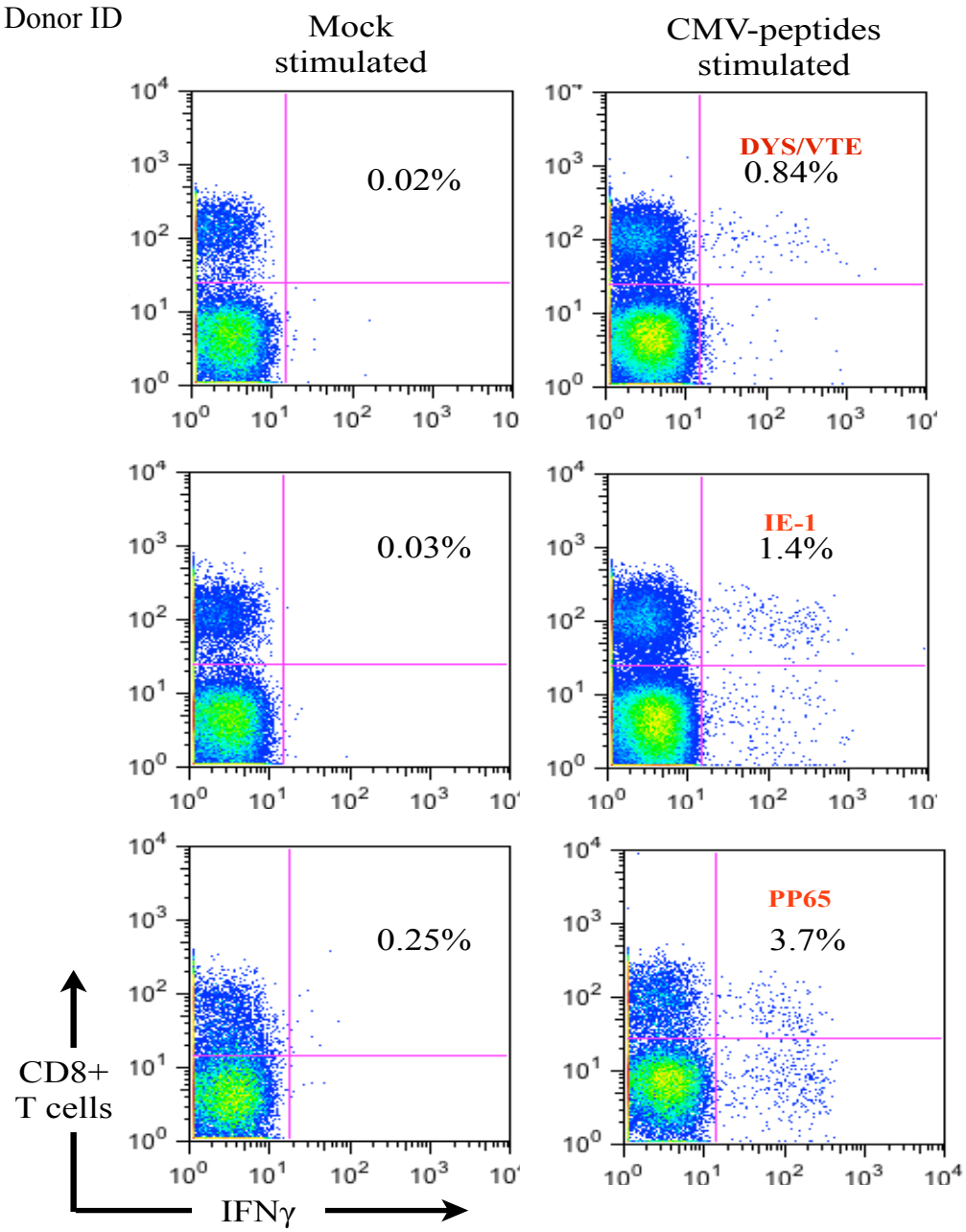


Figure 3.4: CD8+ T cell responses to the different peptide antigens.

Illustrative example of CD8+ T cell responses in a CMV- seropositive healthy donor to three different pools of purified CMV peptides (from top to bottom DYS/VTE, IE-1,and PP65 peptides). The proportion of CMV specific cells is shown as a percentage in the upper right quadrant.

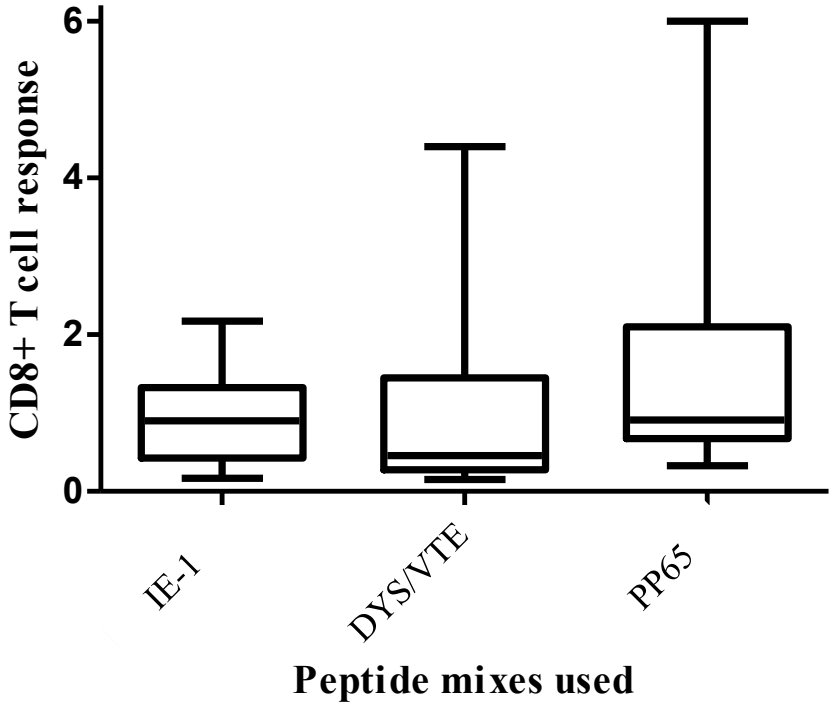


Figure 3.5: Mean of CMV specific CD8+ T cell responses to a mix of synthetic peptides.

This figure demonstrates CMV specific CD8+ T cell responses of 10 CMV-seropositive donors to synthetic peptides (after subtracting the mock level). The highest response is toward pp65 peptides, followed by response to IE-1 peptides and to a lesser extent to DYS/VTE peptide mixes (mean= 1.68 with S.D= 1.819, mean= 0.987 with S.D=1.3 and mean= 0.933 S.D=0.59 respectively).

3.4.2 Phenotyping

The influence of CMV seropositivity on the immune system of immunocompetent subjects has been and is still an area of research interest. Most studies focused on changes in each arm of the immune system separately. This includes individually studying the phenotype of CD4+ and CD8+ T cells, NK cells or to a lesser extent γδ T cells. However, far too little attention has been paid to investigating such changes in the same cohort at the same time. Therefore, to have a comprehensive view, the current study was designed to compare simultaneously differences in frequencies of certain cell phenotypes in CMV positive and CMV negative healthy donors. This includes $CD28^{neg}$ CD4+ T cells, $CD28^{neg}$ CD8+ T cells, $NKG2C^{pos}$ NK cells and $V\delta2^{neg}$ γδ T cells.

3.4.2.1 Possibility of using frozen PBMC samples for Phenotyping

During the initial optimization stages, it was worth investigating the possibility of using frozen PBMC samples instead of fresh blood each time. For this purpose six samples of blood from the same donor were used, three of the samples were fresh and three were recently frozen (within 10 weeks duration). Each sample was tested for both surface cell markers and response to antigens as explained above. A representative example is shown in Figure 3.6. Herein, the CD8+ T cell response to synthetic peptides was measured using flow cytometry and intracellular staining to detect any increase in IFNγ production after stimulation. No significant difference in antibody surface staining assay was detected. However, the functional assay was slightly weaker in frozen samples. Thus a decision was made to use fresh blood samples or recently frozen samples for all functional assays. Meanwhile old frozen cells might be used for non-functional assays such as antibody staining of cell surface markers.

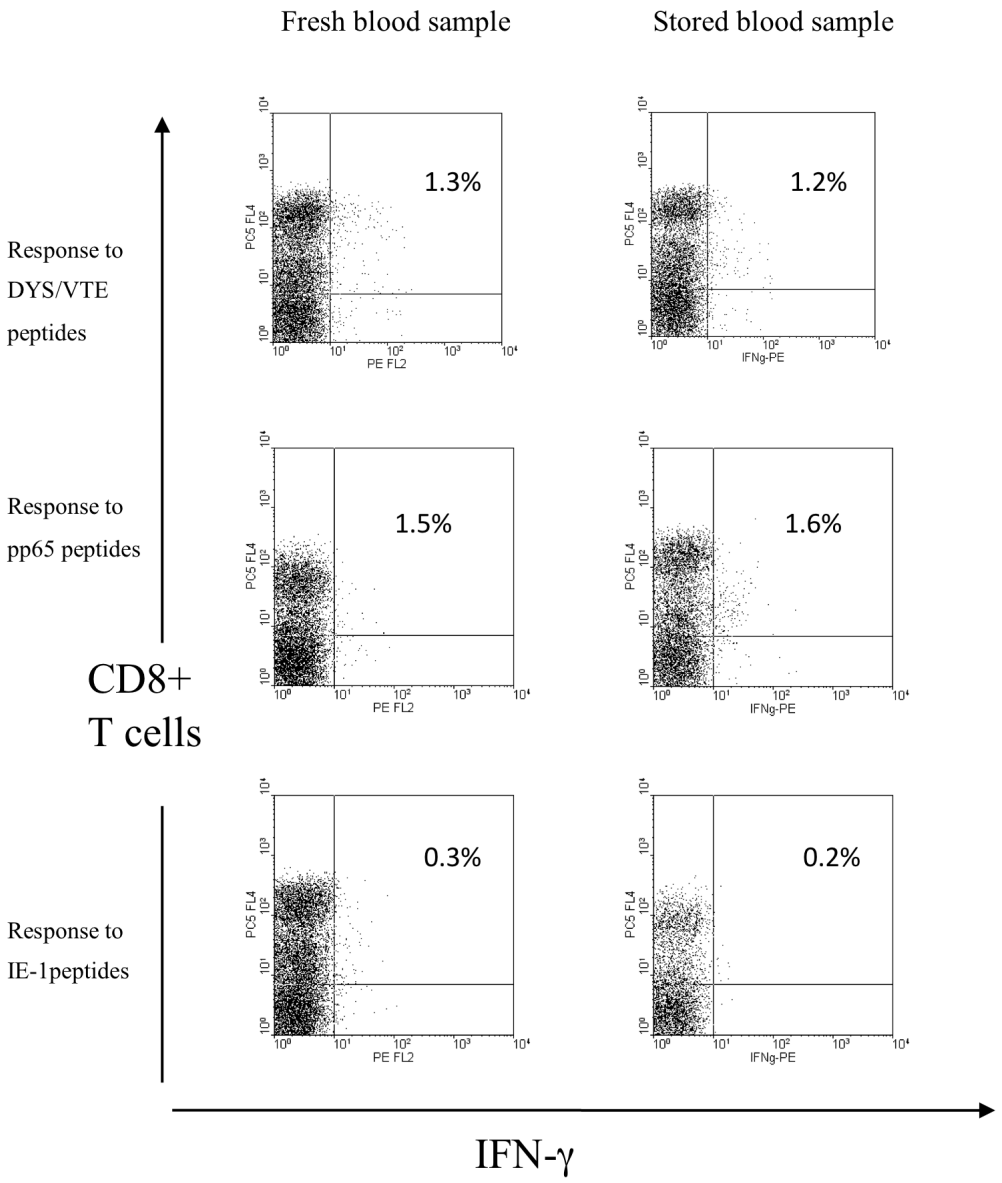


Figure 3.6: Examples of CD8+ T cell responses in fresh and frozen PBMC samples

This figure shows comparison between the response of fresh and recently frozen CD8+ T cells to different synthetic CMV peptides. The stored samples were cryopreserved below -70°C for approximately 6 weeks. From the same donor a fresh blood sample was taken and used as a control for the stored sample. As can be seen there is only a slight difference between the fresh and recently frozen cell responses to the antigens.

3.4.2.2 Higher frequency of CD28 negative CD4+ and CD8+ T cells in CMV-seropositive compared to CMV-seronegative donors.

The role of CD28 is well established in promoting T cell proliferation, survival, metabolism and cytokine production; it is reviewed in detail by Acuto and Michel (Acuto and Michel, 2003). The down regulation of its expression by repeated stimulation makes it one of the indicative markers for T cell differentiation (Hamann *et al.*, 1997, Appay and Rowland-Jones, 2004, Appay *et al.*, 2008). A representative example of how these subpopulations were identified is shown in Figures 3.7 and 3.8. Briefly, plotting size (forward scatter, FSC) versus granularity (side scatter, SSC) was used to identify the lymphocyte population. Upon gating on lymphocytes CD4+ and CD8+ T cell subpopulations were then identified. Finally, according to expression of CD28, both CD4+ and CD8+ T cells were dissected into early ($CD28^{pos}$ or $CD28^{high}$) and late ($CD28^{neg}$ or $CD28^{low}$), also called highly differentiated T cells. The differences in the frequency of these subsets of T cells in CMV-seropositive and CMV-seronegative were compared in the charts below the flow cytometric plots in Figures 3.7 and 3.8.

There was a significant ($p< 0.001$) increase in the frequency of $CD28^{neg}$ CD4+ T cells in CMV-seropositive donors (9.6, 2.8-15.8, *n*=34; median, 25th-75th percentile) compared to CMV-seronegative donors (0.98, 0.59-4.96, *n*=24; median, 25th-75th percentile). Similarly, there was a significant difference ($p< 0.001$) in the percentage of $CD28^{-neg}$ CD8+ T cells between CMV-seropositive (36.48, 29.25-41.50, *n*=34; median, 25th-75th percentile) and CMV negative groups (16.5, 11.64-36.85, *n*=24; median, 25th-75th percentile).

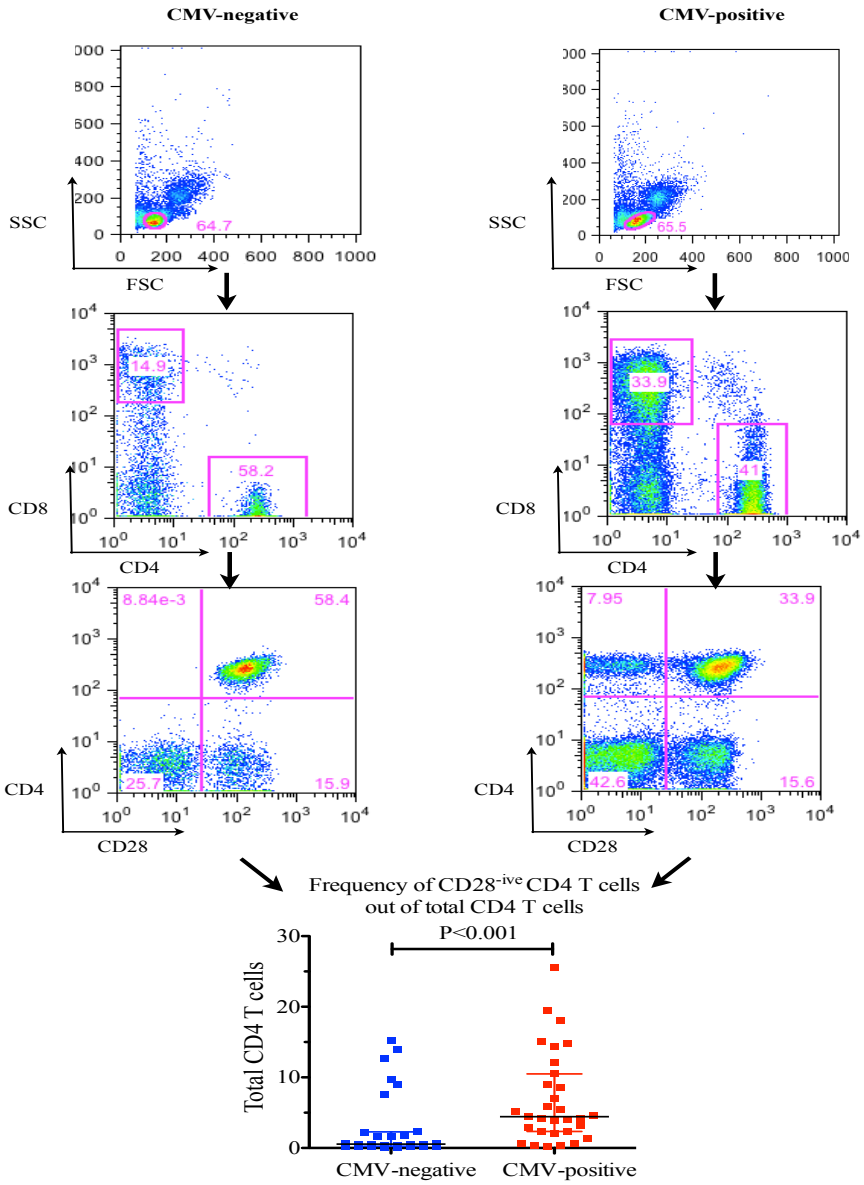


Figure 3.7: Phenotypic profile of CD4+ T cell subsets in CMV-seropositive and CMV-seronegative donors.

The figure shows representative data of flow cytometry analysis from CMV-seronegative (left column) and CMV-seropositive donors (right column). Forward vs. side scatter was used to identify lymphocytes followed by gating on CD4+ and CD8+ T cells. Finally the percentage of $CD28^{neg}$ CD4+ T cells was calculated (the number within the upper left quadrant). The Chart below the FACS-plots summarised $CD28^{neg}$ phenotypic profiles of CD4+ T cells in CMV-seropositive and CMV-seronegative donors. Error bars indicate the median, 25th, and 75th percentile (9.6, 2.8 -15.8, *and* 0.98, 0.59-4.96; CMV-seropositive and CMV-seronegative respectively) with P<0.001 at 95% confidence intervals are shown.

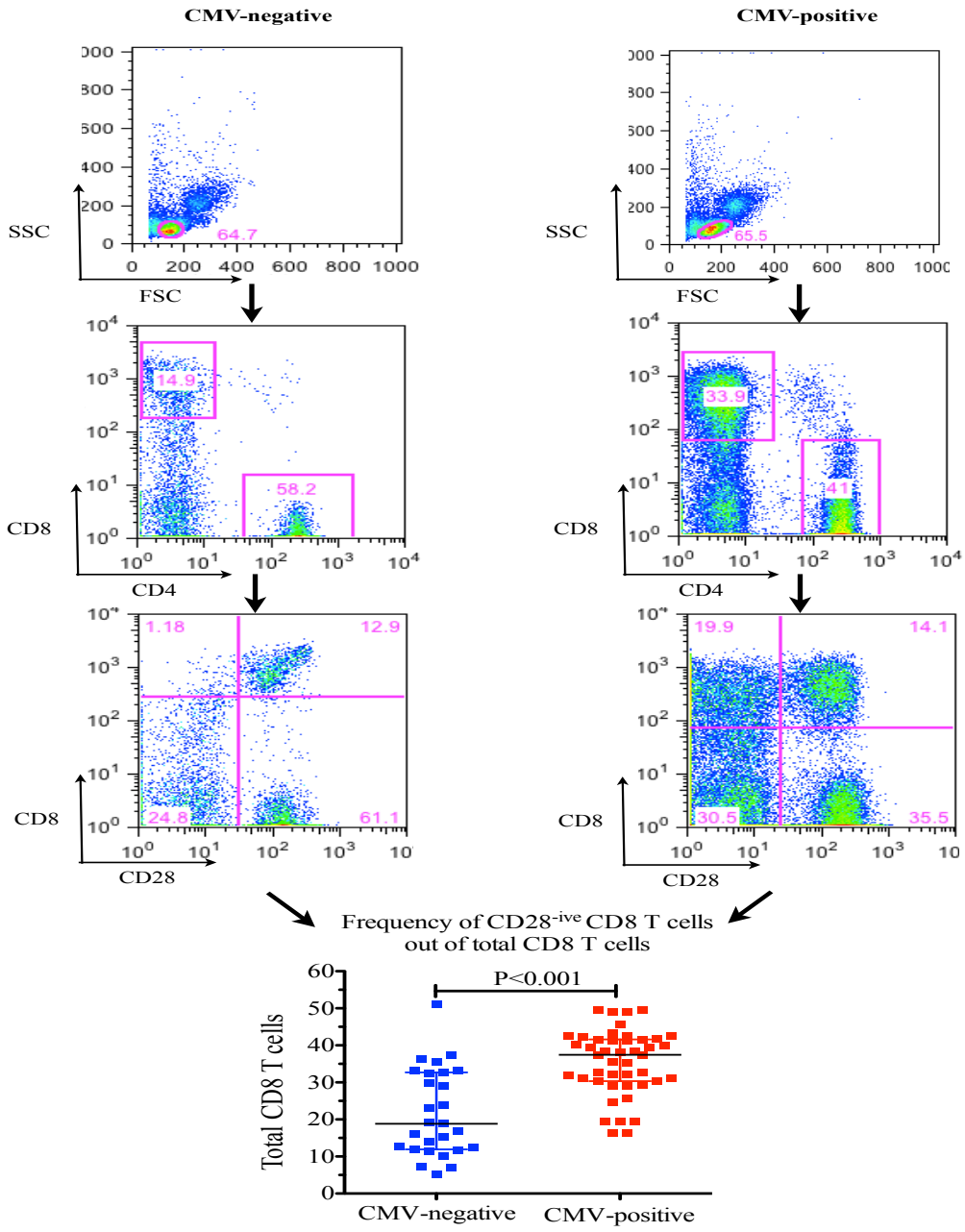


Figure 3.8: Phenotypic profile of CD8+ T cell subsets in CMV-seropositive and CMV-seronegative donors

The figure shows representative data of flow cytometry analysis from CMV-seronegative (left column) and CMV-seropositive donors (right column). Frequency of $CD28^{neg}$ CD8+ cells was calculated and summarised in the chart. There was a significant difference ($p< 0.001$) in the percentage of $CD28^{neg}$ /CD8+ T cells between CMV positive group (36.48, 29.25-41.50, *n*=34 median, 25th-75th percentile) and CMV negative groups (16.5, 11.64-36.85, *n*=24 median, 25th-75th percentile).

3.4.2.3 Similarity in the frequency of total NK cells in CMV positive and CMV negative subjects with remarkable expansion of NKG2C^{pos} NK cells in CMV positive donors.

Aiming to determine the influence of chronic CMV infection on NK cells, two levels of flow cytometric analysis were carried out. Initially, the frequency of immature and mature NK cells among the lymphocyte population was identified. Thus after gating on lymphocytes the CD3^{neg}CD56^{pos} population were selected, which subdivide according to expression of CD16 into immature and mature NK cells (CD16^{neg} NK cells and CD16^{pos} NK cells respectively), see figure 3.9. Comparison between CMV positive and CMV negative subjects showed similarities in the frequencies of both immature NK cells (not shown) and mature NK cells (Figure 3.9).

Indeed, such similarity in frequency of total NK cells does not necessary mean similarity in frequency of all minor subtypes of NK cells. Therefore, a second step was carried out to compare certain subsets of NK cell. Though there are several activating and inhibitory receptors involved in NK cell CMV immunity, in this thesis, NKG2C was chosen. This is based on several observations that suggest the importance of NKG2C^{pos} NK cell in CMV infection (Guma *et al.*, 2004, Guma *et al.*, 2006, Monsivais-Urenda *et al.*, 2010). Figure 3.10 is an illustrative example of gating steps to determine this population. Statistical analysis confirms the presence of a significantly high frequency of NKG2C^{pos} NK cells in CMV positive compared to CMV negative donors ($p<0.05$) at 95% confidence intervals.

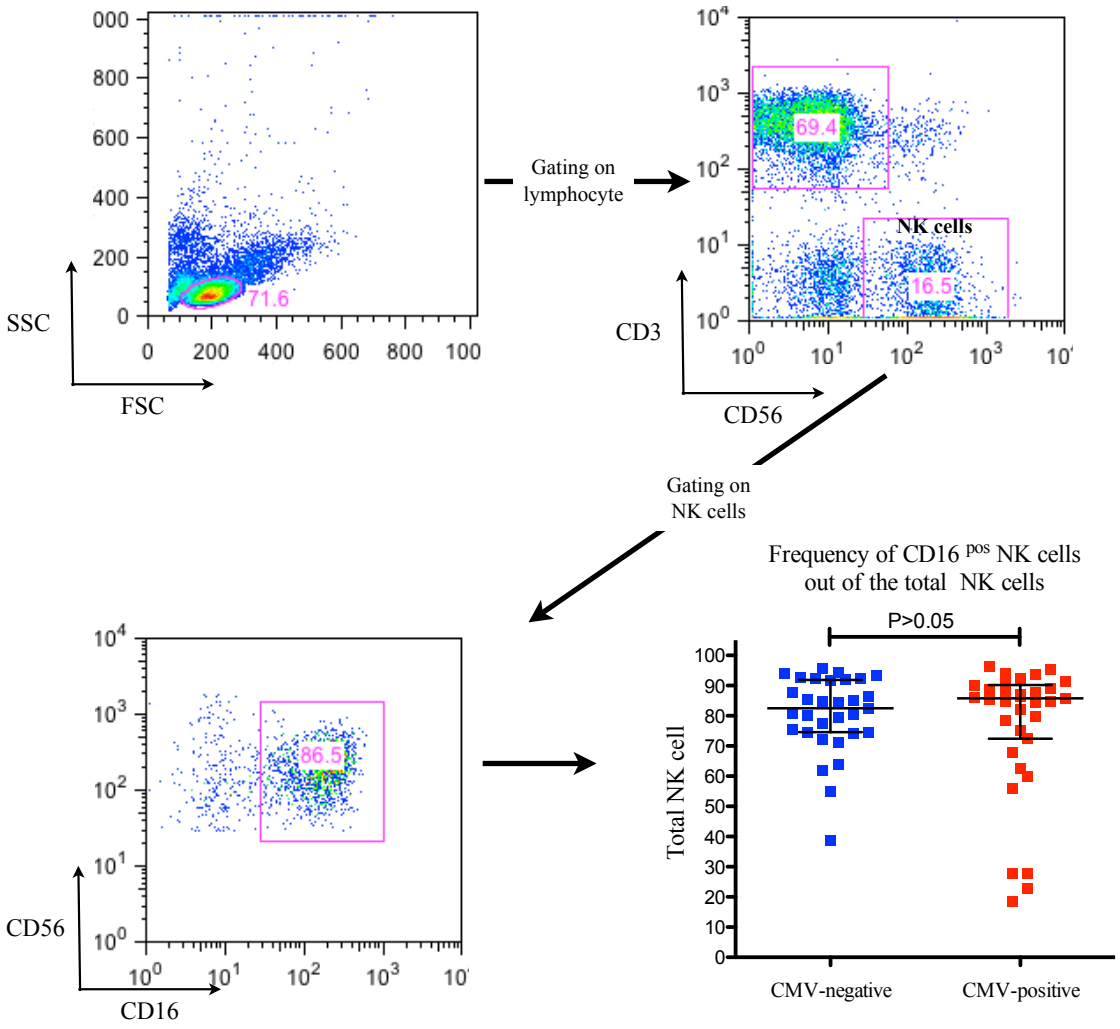


Figure 3.9: Similarity in mature NK cell frequency in CMV-positive and CMV-negative donors.

NK cells were classified according to expression of CD16 into mature ($CD16^{pos}$ NK cell) and immature NK cells ($CD16^{neg}$ NK cells). The frequency of mature NK cell populations in both the CMV positive and CMV negative healthy donors were compared in the chart.

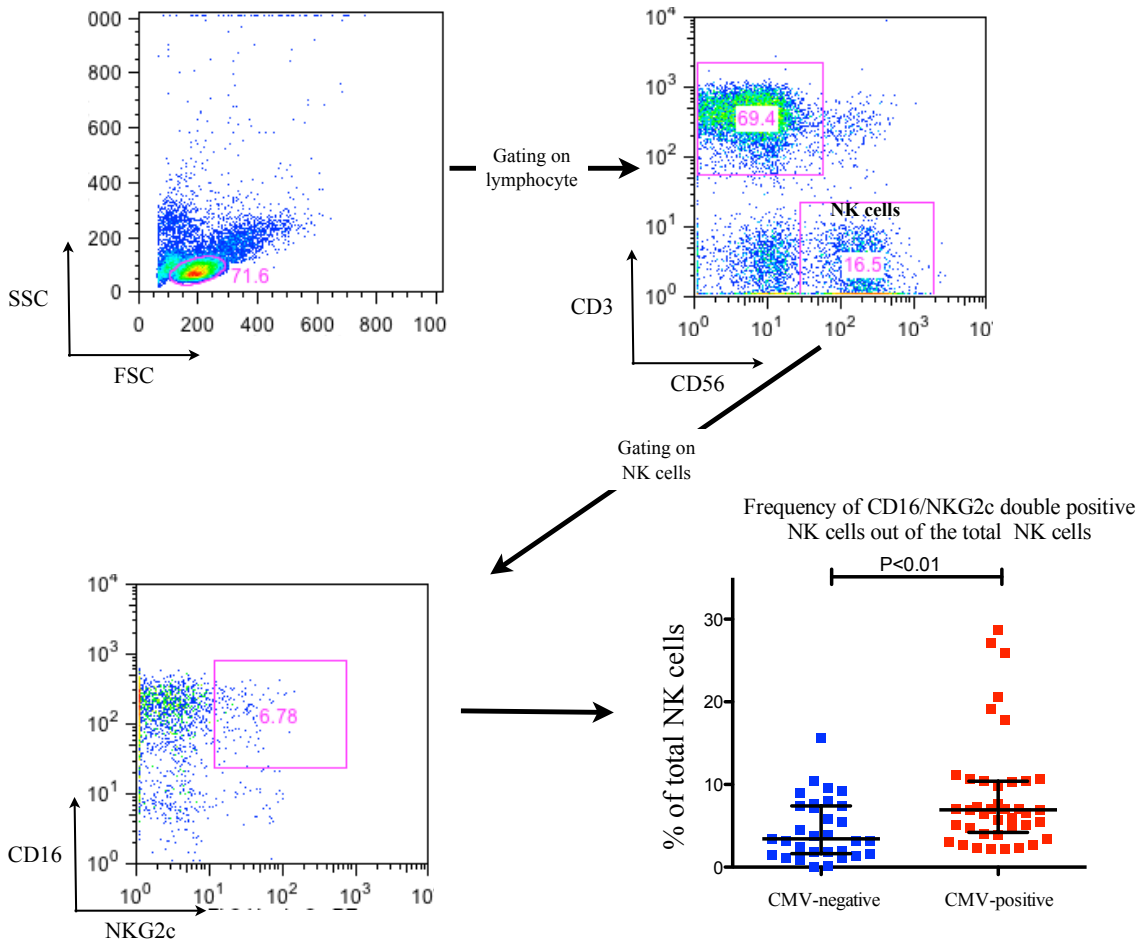


Figure 3.10: High frequency of NKG2C^{pos} NK cells is detected in CMV-seropositive donors.

Flow cytometric plots show gating steps to determine NKG2c^{pos} mature NK cells (CD3^{neg}CD56^{pos} CD16^{pos} NK cells). Diagram shows frequencies (median, 25th-75th percentile) of NKG2C^{pos} NK cells among total NK cell populations in CMV-seropositive (6.93, 4.193-10.40) (red squares) and CMV-seronegative donors (3.43, 1.64-7.40) (blue squares). Error bars signify the median and interquartile and significance (p) values at 95% confidence intervals are shown at the top of the diagram.

3.4.2.4 Remarkable increase in the frequency of Vδ2^{neg} γδ T cells in CMV-positive donors compared to CMV-negative donors.

The role of γδ T cells, particularly the Vδ2^{neg} subset in relation to CMV in healthy subjects has been studied relatively recently (Pitard *et al.*, 2008). Meanwhile, several studies were carried out on immunosuppressed patients with active CMV disease such as renal transplant and bone marrow transplant (BMT) patients (Dechanet *et al.*, 1999a, Knight *et al.*, 2010). Therefore, the aim of the current study was firstly to confirm the expansion of this cell subset in a healthy donor cohort in correlation to CMV status. Secondly to find out if there is redundancy between Vδ2^{neg} γδ T cells and CMV-specific αβ T cells and NK cells.

To determine if there is any difference in γδ T cells or in the proportions of the subclass Vδ2^{neg} γδ T cells between CMV-seropositive and CMV-seronegative donors, PBMC were analyzed by flow cytometry. Here PBMC were stained with antibodies against αβ-TCR, pan-γδ-TCR and Vδ2-TCR using 3-colour staining. A representative example of the gating steps is shown in Figure 3.13. Statistically, there was a significant increase in the proportion of Vδ2^{neg} γδ T cells (P value < 0.0001) in CMV seropositive compared to CMV seronegative healthy donors, see chart in Figure 3.11. However, there was no statistically significant difference (P value < 0.1047) in the proportion of total γδ T cells between groups (not shown).

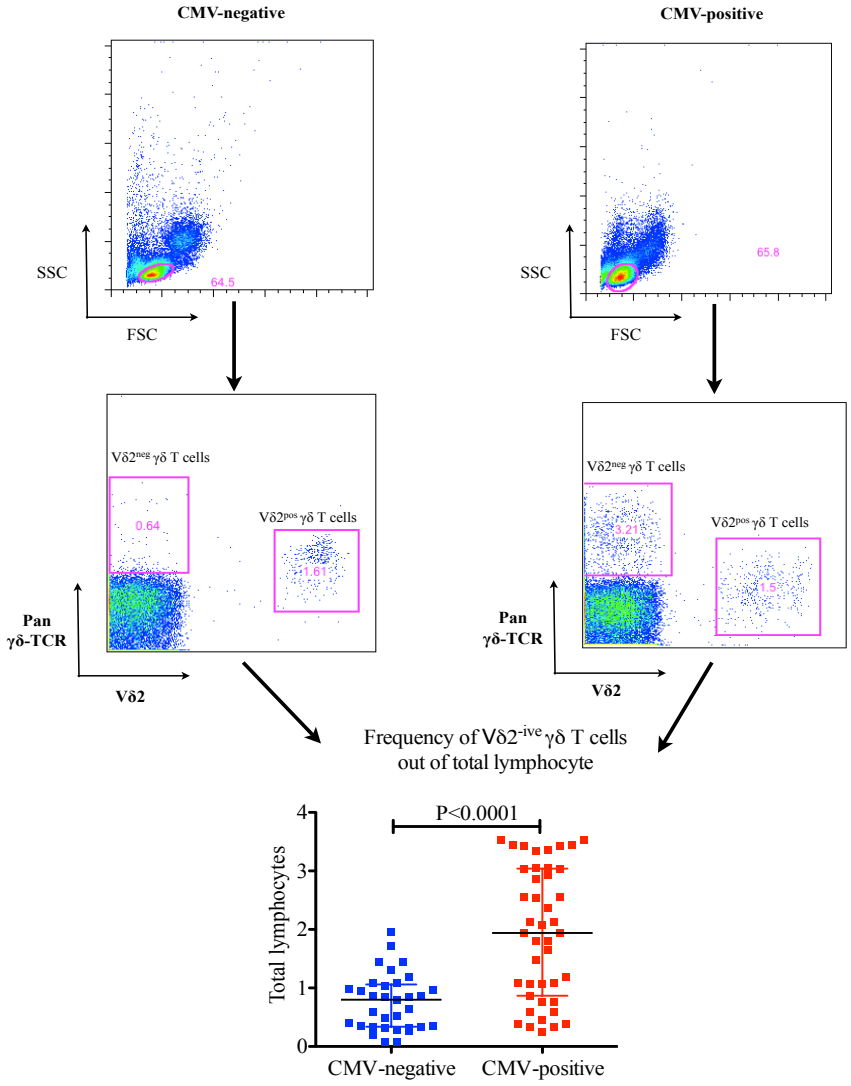


Figure 3.11: Remarkable increase in the frequency of Vδ2^{neg} γδ T cells in CMV-positive compared to CMV-negative healthy donors.

Flow cytometric plots are representative examples of characterization of Vδ2^{neg} γδ T cells from CMV-seronegative and CMV-seropositive healthy donor (left plots and right plots respectively). Gates were designed to show the frequency of Vδ2^{neg} γδ T cells out of total lymphocytes, which are then represented in the chart as median and 25th-75th percentile. In CMV-seropositive (red squares) the frequency was (1.94, 0.81-2.98) while in CMV-seronegative donors (blue squares) it was (0.633, 0.33-1.94) Error bars indicate median with interquartile and significance (p) values at 95% confidence intervals are shown at the top of the graph.

3.4.3 These changes in the distributions of lymphocyte subsets are characteristic of CMV chronic infection in healthy donors.

Aiming to determining whether these phenotypic changes are attributed to CMV infection and not general phenomena of any persistent viral infection, the impact of herpes simplex virus (HSV) carrier status on these subsets of lymphocytes was investigated. therefore, sera from the study cohort (CMV positive and CMV negative groups) were tested by ELISA for the presence of anti-HSV-1 and -2 IgG antibodies (see section 2.3.1.4). As a result there were four groups of healthy donors; CMV^{pos} HSV^{pos} (n=29), CMV^{pos} HSV^{neg} (n=12), CMV^{neg} HSV^{pos} (n=17), and CMV^{neg} HSV^{neg} (n=14). Frequencies of lymphocytes of interest were compared among these groups of donors (Figure 3.12).

Comparing the healthy donors according to HSV status (i.e. CMV^{pos} HSV^{pos} vs. CMV^{pos} HSV^{neg} and CMV^{neg} HSV^{pos} vs. CMV^{neg} HSV^{neg}) showed no significant difference in the frequency of all kinds of cells of interest. Meanwhile, comparing cells according to CMV status such as, CMV^{pos} HSV^{pos} vs. CMV^{neg} HSV^{pos} demonstrated remarkable differences in frequencies of all kinds of lymphocytes of interest. However comparison between CMV^{pos} HSV^{neg} and CMV^{neg} HSV^{neg} groups showed differences only when considering frequency of $CD28^{neg}$ CD4+ T cells and $V\delta2^{neg}$ γδ T cells.

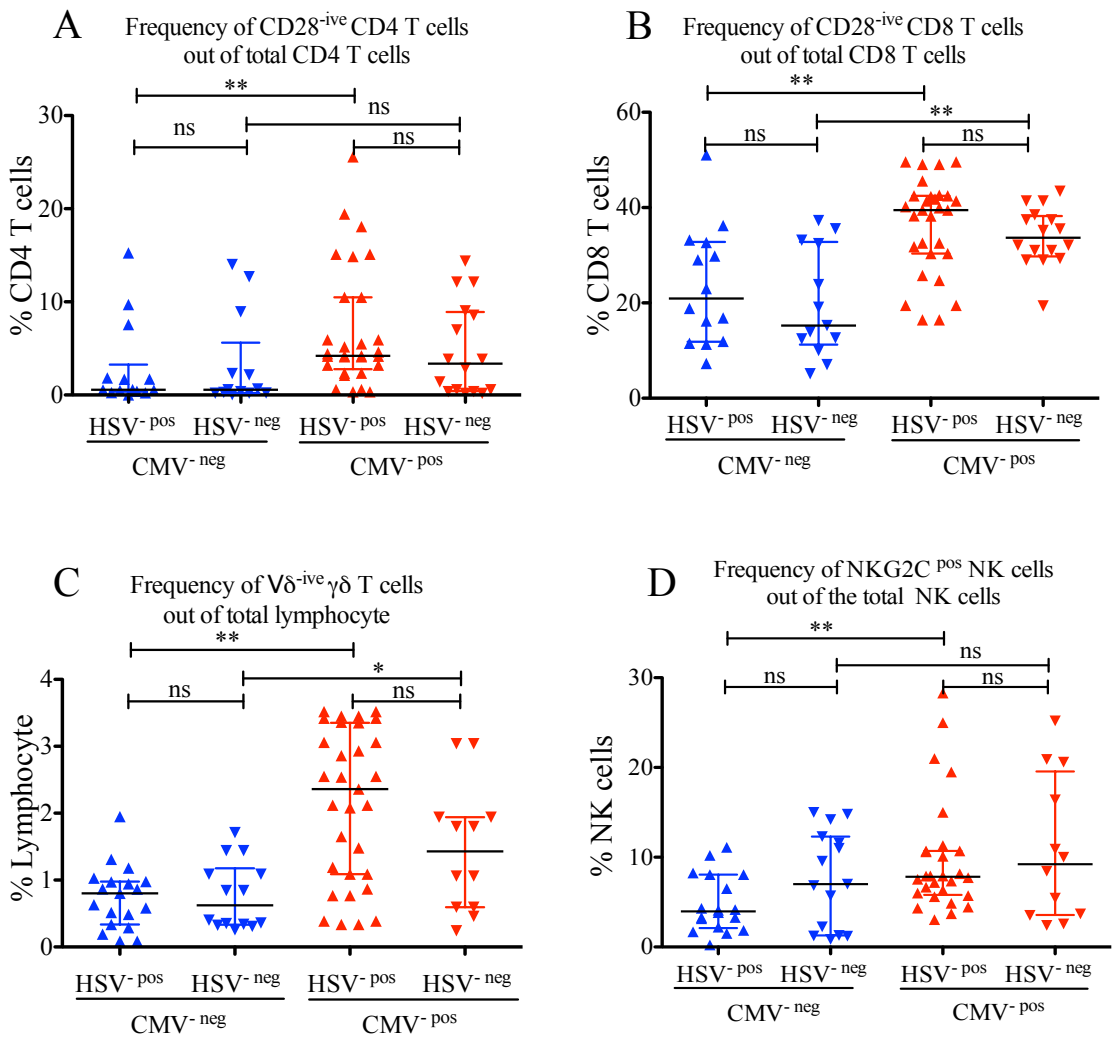


Figure 3.12: The impact of HSV infection on the distributions of different lymphocyte subsets in CMV- seropositive and CMV–seronegative healthy donors.

The study population was divided according to CMV and HSV serology into four populations; CMV^{pos} HSV^{pos} (n=29), CMV^{pos} HSV^{neg} (n=12), CMV^{neg} HSV^{pos} (n=17), and CMV^{neg} HSV^{neg} (n=14). Infection with CMV but not HSV induced the accumulation of late differentiated effector αβ T cells ($CD28^{neg}$ CD4+ T cells and $CD28^{neg}$ CD8+ T cells) chart A and B respectively. Also the increase in frequencies of $V\delta2^{neg}$ γδ T cells and $NKG2C^{pos}$NK cells was related to CMV seropositivity but not HSV status (see chart C and D). Significance (p) values at 95% confidence intervals are shown as asterisks at the top of the graph (ns= not significant, *= $p<0.05$, **= $p<0.001$, and ***= $p<0.0001$).

3.4.4 Dynamic monitoring of phenotypic changes

Comparing the lymphocyte phenotype in CMV seropositive to CMV seronegative subjects provided only a view on the differences in the immune system pre and post infection with a gap in the knowledge of how long these changes had taken to develop. Therefore a special type of ELISA was carried out to attempt to define the CMV-seropositive cohort into recently infected and durably (long-standing) infected subjects. Basically this assay works by detecting the avidity of CMV-specific IgG and hence donors with low IgG avidity can be considered as CMV-recent infected whereas those with anti-CMV IgG high avidity they must have had CMV for a long period (CMV long-standing infection), see section (2.3.1). According to this assay only three donors in the current study showed low avidity. Indeed, with such low numbers, statistically, it was not possible to compare the two groups. Thus another approach was tried, which was studying changes in the immune system during CMV symptomatic primary infection.

3.4.4.1 Acute symptomatic primary CMV infection in immunocompetent patients.

Studying CMV symptomatic primary infection is usually carried out on immune-compromised patients such as post renal transplantation where there is a high incidence of CMV infection. Nevertheless because the population of this study was recruited from healthy donors, identification of symptomatic CMV primary infection of immune-competent subjects was tried. Since the incidence of CMV symptomatic primary infection in Merseyside hospitals is approximately eight cases per year, the goal was to recruit 4 to 8 patients. However, only one of five patients diagnosed with primary CMV infection yielded samples.

From the consented patient 20 ml of blood was taken at three different time points; T1 blood sample was taken the second day of diagnosis (first day of acyclovir therapy), T2 was taken 10 days after therapy initiation while T3 was taken 6 weeks later. After separation of PBMC in the standard way, plasma was collected and used to measure both CMV specific antibody titres by ELISA and viral load utilizing Real time PCR (see section 2.3.2)

3.4.4.2 Relationship between viral load and CMV-specific IgG antibody titre and changes in composition and frequency of effector αβ T cell subsets

Aiming to determine the association between the anti CMV-IgG and CMV viral load on the one hand and the change in the phenotype of lymphocytes, the viral load and antibody titre were correlated to the frequency of target cell populations. Starting with viral load there was a remarkable drop in the number of viral DNA copies per ml of plasma from $472x10^{-2}$ to $1\ x10^{-2}$ between T1 and T2. However, by T3 viral DNA was not detectable. In contrast, the level of CMV-specific IgG antibody was almost constant and only started to decline by T3 (Figure 3.13).

Dynamically, αβ T cells showed an initial sharp increase in the percentage of $CD28^{neg}$ CD4+ T cells from 3.25% at T1 to 7.56% at T2, followed by a slow increase to reach 7.97% by T3. This level seemed to be approaching that in CMV chronic infection, (see 3.4.2.2, and Figure 3.7), where the median was 9.6 and 25th-75th percentile ranges between 2.8 and 15.8; respectively. In contrast, $CD28^{neg}$ CD8+ T cells demonstrated a remarkable reduction, at T3 to approximately half their frequency at T1 (T1= 61.5% T3= 30.2%). Again this suggests that levels were approaching those of $CD28^{neg}$ CD8+ T cells

in CMV chronic infection (36.48, 29.25-41.50, *n*=34: median, 25th-75th percentile) (section 3.4.3.2 and Figure 3.8).

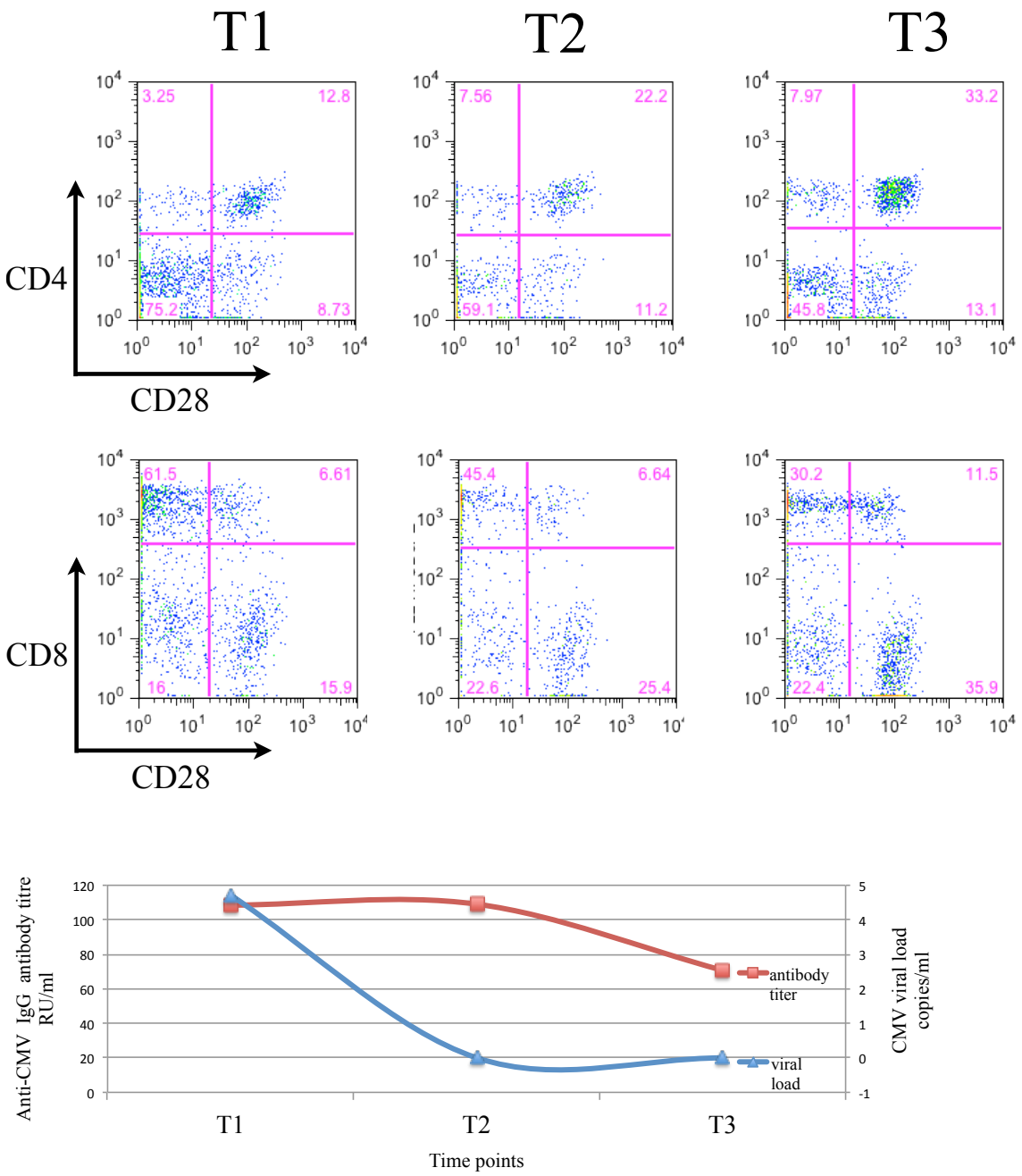


Figure 3.13: Phenotypic changes in CD4+ and CD8+ T cells after primary CMV infection.

Flow cytometric plots from an immunocompetent healthy donor with acute primary CMV infection. T1 blood sample was taken on the second day of diagnosis and first day of acyclovir therapy, T2 was taken 10 days after therapy initiation while T3 was taken 6 weeks later. The upper two panels show the frequency of $CD28^{-neg}CD4+$ and CD8+ T cells as % total lymphocytes (upper left quadrant in each plot). The chart below shows the changes in viral load (blue curve) and anti-CMV IgG antibody titre (red curve).

3.4.4.3 Alteration in the percentage of NKG2C^{pos} NK cells and Vδ2^{neg} γδ T cells after primary CMV infection.

The dynamic changes in the frequency of NKG2C^{-pos} NK cells and Vδ2^{neg} γδ T cells post acute primary CMV infection are illustrated in Figures 3.14 and 3.15 respectively. Herein, changes in the viral load and anti-CMV IgG antibody titre (displayed as blue and red curves, respectively) are plotted against frequencies of these cells at three different time points (T1, T2, and T3). Both NKG2C^{pos} NK cell and Vδ2^{neg} γδ T cell frequencies gradually declined over time. Table 3.1 below summarises the change in the frequency of each subset compared to their corresponding medians (25th-75th percentile) in CMV positive chronic carriers.

Table 3-1 Cell frequencies in CMV infected patient compared to medians and means of healthy CMV+ carriers.

	CMV primary infection			CMV positive chronic carriers	
	T1	T2	T3	Median	25th-75thpercentile
NKG2C^{pos} NK cells	26.9	23.9	21.6	7.9	5.4 - 11.2
Vδ2^{neg} γδ T cells	2.37	1.69	1.42	1.94	0.86 - 3.5

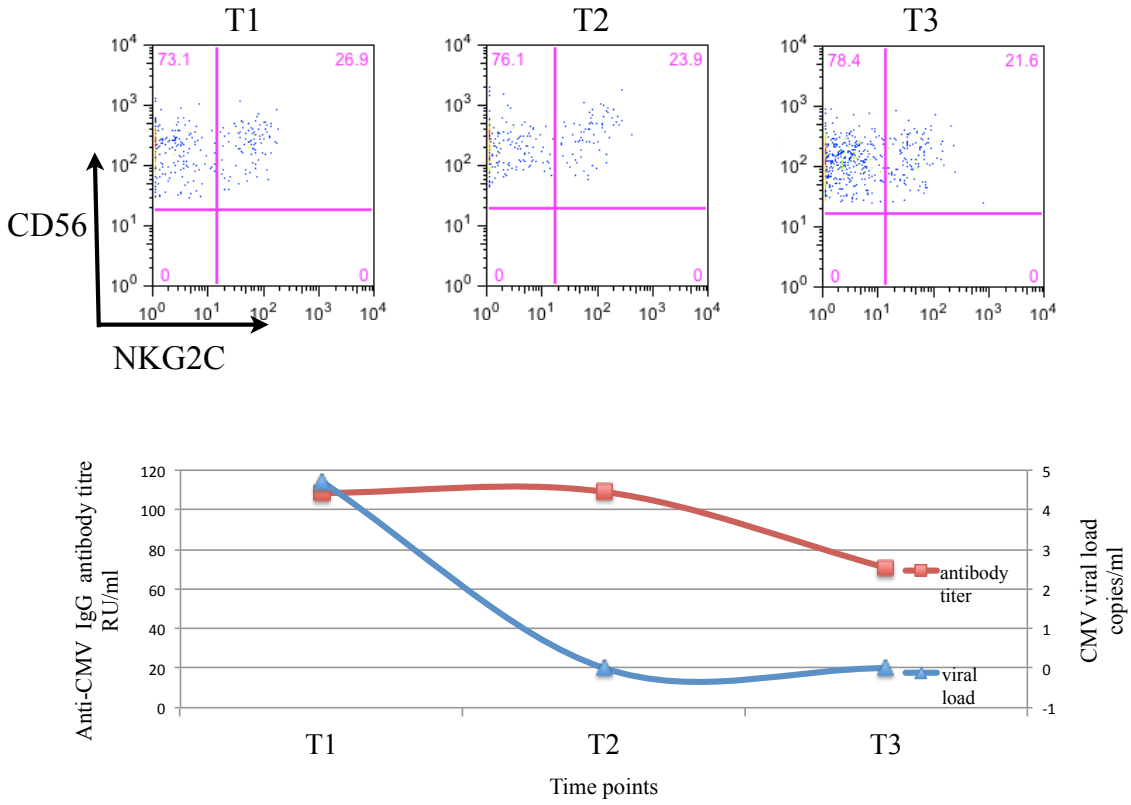


Figure 3.14: Changes in the levels of expression NKG2C in NK cells after primary CMV infection.

Dynamic changes in the frequency of NKG2C^{pos} NK cells (upper right quadrant) post acute primary CMV infection in an immunocompetent healthy donor. Time points T1, T2, and T3 were taken on the second, 10th, and 28th day of diagnosis. The chart below displays the changes in viral load (blue curve) and anti-CMV IgG antibody titre (red curve).

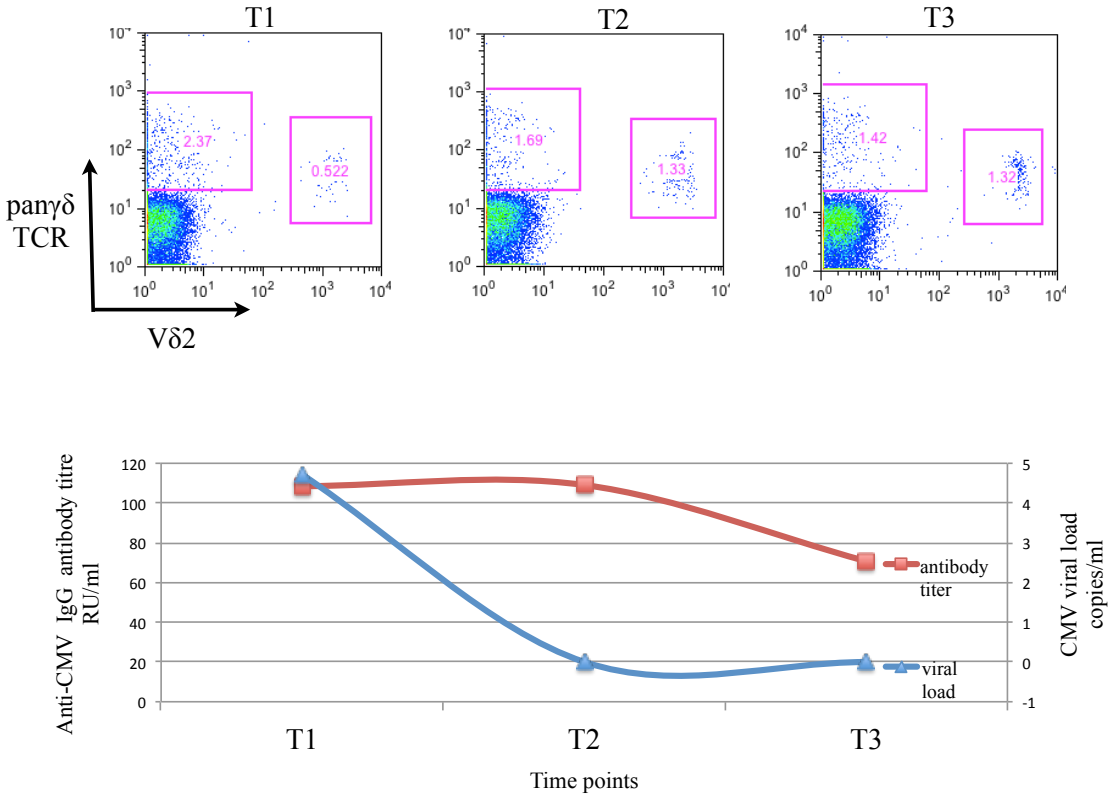


Figure 3.15: Alteration in the percentage of Vδ2^{-neg} γδ T cells after primary CMV infection

The panel of flow cytometric plots demonstrates frequencies of both Vδ2^{pos} (right rectangle gate) and Vδ2^{neg} (left rectangle gate) γδ T cells on the second, 10th, and 28th day post diagnosis of acute primary CMV infection. The chart below shows associated changes in viral load (blue curve) and anti-CMV IgG antibody titre (red curve).

3.4.5 Significant positive correlation between Vδ2^{neg} γδ T cells and CD28^{neg} CD4+ T cells in CMV-seropositive healthy donors.

A regression analysis was used to determine the presence of association (such as co-operation/redundancy) between different lymphocyte subsets, which includes CD28^{neg} CD4+ T cells, CD28^{neg} CD8+ T cells, NKG2C^{pos} NK cells and Vδ2^{neg} γδ T cells, in CMV-seropositive healthy donors. The matrix of correlations was generated by plotting the linear regression with the best-fit line and 95% confidence band between all possible cell subsets combinations. Spearman Correlation coefficient (r) and P values were used to test whether slopes and intercepts are significantly different. The results obtained from this analysis are presented in Figure 3.16.

Starting with the association between αβ T cells and other subsets, while the linear regression analysis showed no significant association between levels of CD28^{neg} CD8+ T cells and Vδ2^{neg} γδ T cells in CMV-seropositive subjects (r = 0.39 with P = 0.177), there was significant positive correlation between CD28^{neg} CD4+ T cells and that of Vδ2^{neg} γδ T cells (r = 0.61 with P = 0.016). In contrast, neither CD4+ nor CD8+ T cells showed significant association with NKG2C^{pos} NK cells (r=0.34,P=0.344 and r=0.18,P=0.347, respectively).

Regarding the association of NK cells with γδ T cells, though the best-fit line of linear regression between NKG2C^{pos} NK cells and Vδ2^{neg} γδ T cells was in a positive direction, statistically there was no significant association between these subsets (r= 0.19 and p=0.463).

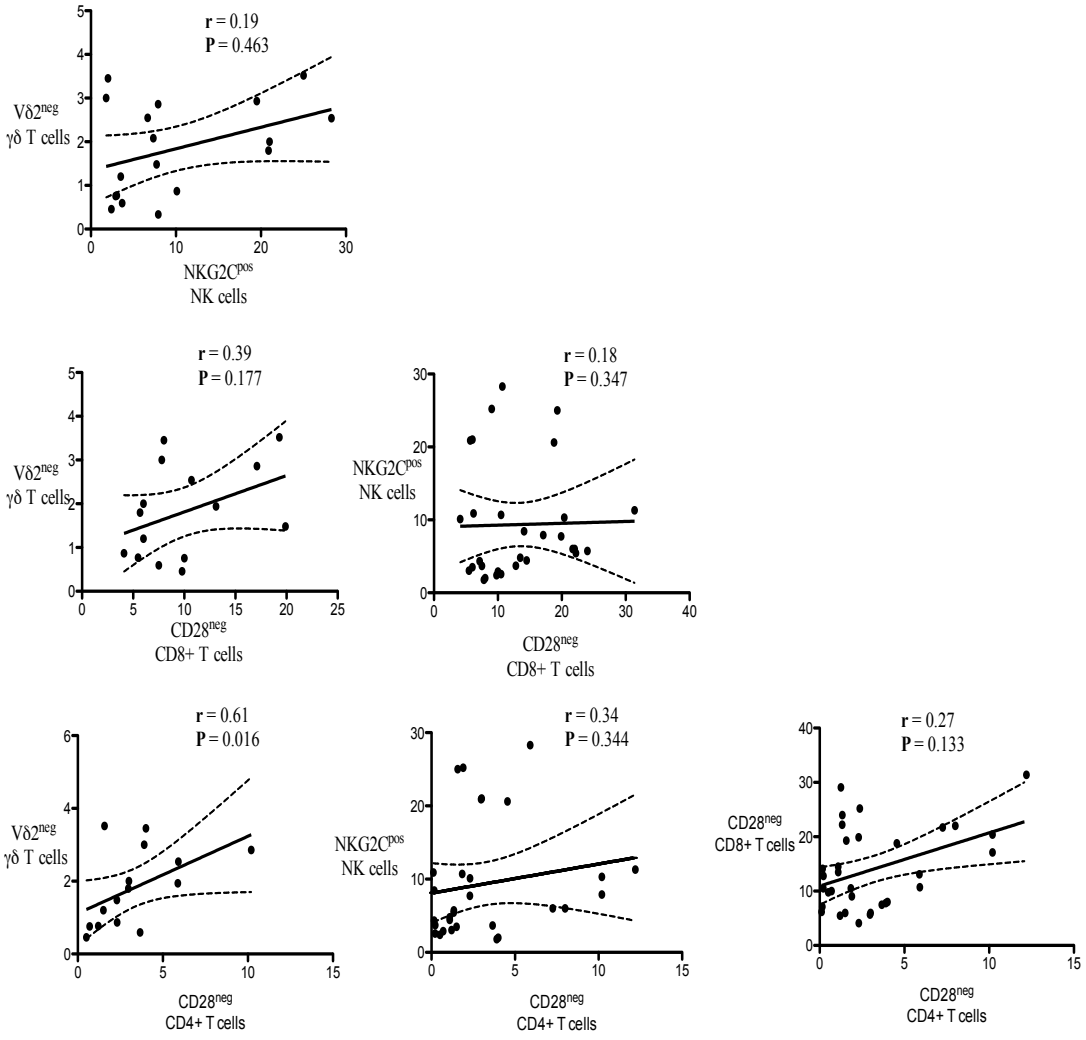


Figure 3.16: Correlations between levels of different lymphocyte subsets in CMV positive immunocompetent donors.

The cluster of charts demonstrates the linear regression between different lymphocyte subsets. All possible combinations of cells subsets were plotted. The lines in the each chart represent the best-fit line with 95% confidence band. Spearman Correlation coefficient (r) and P value are shown at top of each chart.

3.5 Discussion

The aim of this chapter was to investigate the influence of chronic CMV infection on different lymphocyte subsets in immunocompetent donors. This includes examining the concurrent change in the frequency of lymphocyte subsets that are believed to be important in CMV infection. This involved determining the frequency of $CD28^{neg}$ CD4+ and $CD28^{neg}$ CD8+ T cells, $NKG2C^{pos}$ NK Cells and $V\delta2^{neg}$ γδ T cells to find out if there is any association between these subsets. To achieve these aims, 75 healthy donors were recruited and classified according to detection of anti-CMV IgG antibodies and CMV-specific αβ T cell response into CMV negative and CMV positive subgroups (approximately 52% and 48% of cohort; respectively). This was followed by statistical comparison of the frequencies of lymphocytes of interest between both groups.

It has become more evident that chronic CMV infection has substantial influence on αβ T cells; this is particularly true of CD8+ T cells where several reports demonstrate accumulation of monoclonal highly differentiated CD8+ T cells in CMV-seropositive elderly subjects (Looney *et al.*, 1999, Khan *et al.*, 2002b). However, far less attention has been given to assessing the impact of CMV on the CD4+ T cell repertoire compared to CD8+ T cells (Pourgheysari *et al.*, 2007). Therefore, phenotypic analysis of CD4+ T cells was included in the current study. Using a panel of various surface markers such as CCR7, CD45RA, LFA1, CD27 and CD28 might be necessitated for appropriate phenotyping. For instance using these marker can categorize T cells into Naive (T_N), Central Memory (T_{CM}), Effector Memory (T_{EM}), and Effector Memory CD45RA re-expressing (T_{EMRA}) (Appay *et al.*, 2008). However, the limitation in the number of channels available for flow cytometry allowed only the use of one of these markers

(besides the basic markers: CD3, CD4 and CD8). The decision was made to use CD28, as its down regulation seems to be a highly reliable marker for identification of late differentiated cells (Olsson *et al.*, 2000, Wikby *et al.*, 2002, Acuto and Michel, 2003).

Interestingly, the current study showed that the influence of CMV carriage was not only limited to a remarkable increase in the frequency of $CD28^{neg}$ CD8+ cells but was also associated with a significant increase in $CD28^{neg}$ CD4+ T cells. Furthermore, linear regression analysis indicated positive correlation between $CD28^{neg}$ CD4+ T cells and $CD28^{neg}$ CD8+ T cells, though it was not statistically significant. Indeed, this might suggest that these cells do not play a redundant role in controlling latent CMV infection. In other words, both of them might be needed to keep the latent virus under control. Nevertheless, since these cells are highly differentiated, their function and effectiveness in viral infection control is debatable. For instance, while some reports indicate these cell subsets are exhausted and nonfunctional other demonstrated that they could be re-energized to produce robust functional responses (Waller *et al.*, 2007, Chong *et al.*, 2008).

Regardless if these cells are exhausted or not, the question here is whether their expansions are useful? The exact answer to this question is still an area of debate. For instance, some argue that these cells can play a protective role. This is supported by the very low incidence of severe CMV disease in immune competent subjects with expanded CMV-specific T cell clones. Nevertheless, appearance of donor-specific $CD28^{neg}$ CD8+ T cells in transplant recipients was found to be associated with low incidence of acute rejection, which suggests the suppressive function of these cells (Liu *et al.*, 1998, Colovai *et al.*, 2003). However, If CMV-specific CD8+$CD28^{neg}$ cells were

strongly immunosuppressive an increased incidence of CMV viraemia would be expected in subjects with expanded levels of CMV-specific clones. On the other hand, other reports show a correlation between the expression of PD-1, CMV infection and disease in liver transplant recipients (La Rosa *et al.*, 2007) and with CMV viraemia in renal transplant recipients (Sester *et al.*, 2008). Alternatively, $CD8+CD28^{neg}$ cells may simply be anergic rather than actively immunosuppressive.

There are several subclasses of NK cells that are broadly different functionally and phenotypically. In addition to levels of expression of CD56, which can classifying NK cells into $CD56^{dim}$ and $CD56^{bright}$ populations, expression of CD16 is widely used to categorize NK cells into immature ($CD16^{neg}$) and mature ($CD16^{pos}$) cells (Caligiuri, 2008). The present study showed no differences between CMV-positive and CMV-negative donors regarding the frequencies of both mature and immature NK cells. Though there are several activating and inhibitory receptors that might be involved in NK cell-CMV immunity, $NKG2C^{pos}$ NK cells were chosen. This was based on several observations that suggest the importance of this subset in CMV infection (Guma *et al.*, 2004, Guma *et al.*, 2006, Monsivais-Urenda *et al.*, 2010). Although the exact mechanism how $NKG2C^{pos}$ NK cells can recognize CMV-infected cell has not been fully uncovered, the recognition of HLA-E associated with CMV infection can be proposed. This based on observation showed the proliferation of $NKG2C^{pos}$ NK cells in the presence of IL-15 and HLA-E. Indeed future studies are required to determine if NKG2C directly recognizes CMV-infected cells and if this recognition is HLA-E–dependent.

Interestingly, a recent study has shown that the $NKG2C^{pos}$ NK cells preferentially increased during acute CMV infection; furthermore it demonstrated that this NK cell

subset gradually acquired a CD57+ phenotype (Lopez-Verges *et al.*, 2011). Considering that this phenotype is also expressed by CMV-specific B cells and T cells they are proposed to acquire immunological memory after CMV viral infection. Thus, it is proposed that CD57 might provide a marker of "memory" NK cells that have been expanded in response to infection (Lopez-Verges *et al.*, 2011).

Nevertheless, statistically, there was no association between these cells and other lymphocyte subsets. Indeed such observations highly suggest that NKG2C^{pos} NK cells play a non-redundant role in controlling CMV chronic infection.

There is growing evidence that non-conventional T-cells expressing gamma-delta (γδ) TCR are important in protection against CMV, an example is the role of Vδ2^{neg} γδ T cells in controlling active CMV infection in immunosuppressed patients (Dechanet *et al.*, 1999b). Nevertheless, far too little attention has been paid to their role in CMV infection in immunocompetent subjects. Therefore, one of this study's aims was to investigate the importance of Vδ2^{neg} γδ T cells in controlling CMV in healthy immunocompetent subjects and to find out if their frequency correlates with other lymphocyte subsets in carriers.

The results of this study demonstrate the presence of a remarkable increase in Vδ2^{neg} γδ T cells frequencies in healthy CMV-seropositive donors compared to CMV-seronegative donors. Furthermore it showed the presence of an association between Vδ2^{neg} γδ T cells and αβ T cells, which showed significant positive correlation in case of CD28^{neg} CD4+ T cells. This might suggest the presence of cooperation between the two subsets in

controlling CMV. In other words there is a possibility that activated Vδ2^{neg} γδ T cells might in some way induce proliferation and differentiation in naıve αβ T cells through presenting CMV antigen (or via cytokine production). This notion can be supported by previous studies on Vδ2^{pos} γδ T cells (a major sub-population of γδ T cells) that demonstrated the ability of these cells to professionally present antigen directly to CD4+ T cells through up regulation of HLA class II molecules (Brandes *et al.*, 2005) or to CD8+ T cells through cross-priming (Brandes *et al.*, 2009, Meuter *et al.*, 2010). In the case of Vδ2^{neg} γδ T cells, more investigations are needed to prove or disprove this possibility.

Up to this point, a remarkable change in the composition of lymphocyte subsets was demonstrated in CMV positive healthy donors. This included a significant increase in the frequency of CD28^{neg} CD4+ T cells, CD28^{neg} CD8+ T cells, NKG2C^{pos} NK cells and Vδ2^{neg} γδ T cells in CMV seropositive healthy donors. Nonetheless, having observed such changes in lymphocyte phenotype it was further investigated whether these changes were specific for chronic CMV infection or part of a general phenomenon that can occur with any persistent viral infection, particularly other herpes viruses. To answer this question the impact of herpes simplex virus (HSV) carrier status on these subsets of lymphocyte was investigated. The current study suggested that infection with CMV was most likely the cause of accumulation of late differentiated effector αβ T cells (CD28^{neg} CD4+ T cells and CD28^{neg} CD4+ T cells) as well as being responsible for accumulation of Vδ2^{neg} γδ T cells and NKG2C^{pos} NK cells. Meanwhile, HSV chronic infection could have a similar effect in advanced age.

Ideally, to monitor dynamic change in the phenotype that might be related to an infectious agent, the exact time of acute infection is needed to be known. However, since in most cases CMV infection is subclinical it would be impossible to exactly define the time of acute infection. Therefore, determining the avidity of CMV- specific IgG by a special type of ELISA was carried out. Accordingly, CMV seropositive donors could be classified into two groups, donors with low avidity (recent infection) and high avidity (long-standing infection). In the current study only three donors showed low avidity therefore statistically it was not appropriate to perform accurate comparison between the low and high avidity groups. Thus another approach was tried, which was studying changes in the immune system during symptomatic primary CMV infection. This approach is usually carried out in studies of immune-compromised patients such as post renal transplantation where there is high incidence of CMV infection. Indeed, studying the immune system in immune-compromised patient would not be the perfect module for studying the immune system of healthy subjects. Therefore the use of immune-compromised donors was excluded from the current study. Instead, recruiting of healthy donors with symptomatic primary CMV infection was tried. Based on the incidence of symptomatic primary CMV infection in Merseyside hospitals, which was approximately eight cases per year, the aim was to recruit 4 to 8 patients. Unfortunately, only one out five patients diagnosed with primary CMV infection agreed to participate in the study.

The striking result obtained from this patient was that the change in the phenotype of lymphocytes is related to the change in anti-CMV-IgG titre rather than CMV viral load. In addition, apart from $CD28^{neg}$ CD4+ T cells, which started to appear after viral clearance, all other cell subsets seem to be involved since the beginning of acute

infection. It is crucial to appreciate that this finding from a single patient might not reflect the real kinetics of the immune response during acute CMV infection. Nevertheless, shortage of information in this field made it reasonable to report this result.

4 The Impact Of Age And Cytomegalovirus Carriage On γδ T Cells In Immunocompetent Subjects

4.1 Background

The results from the pilot study that was presented in Chapter 3 demonstrated that chronic CMV-carriage is associated with remarkable changes in the host immune system. This included alteration in phenotype and repertoire of NK cells, αβ T cells and γδ T cells. While the roles of αβ T cells, including CD4+ and CD8+ T cell in CMV immune responses have been studied in great detail (Wills *et al.*, 1996b, Kern *et al.*, 1999, Elkington *et al.*, 2003), there is insufficient information about the role of other T cell subsets. Currently there is increasing appreciation of the role of non-conventional T cells expressing the gamma delta (γδ) TCR in protection against CMV. Furthermore, γδ T cells were found to expand or become activated in infections and in malignancy (Maeurer *et al.*, 1996, Behr-Perst *et al.*, 1999, Wang *et al.*, 2001, Bonneville and Fournie, 2005, Ebert *et al.*, 2006), and also differentiate into memory cells much earlier in life than αβ T cells, indicating a major role in responding against pathogenic insults from birth (De Rosa *et al.*, 2004).

Normally, γδ T cells constitute a minority of circulating T cells and fall into two main subclasses: Vδ2^{pos} γδ T cells (50-90% of γδ T cells, predominant in blood) and Vδ2^{neg} γδ T cells, which include Vδ1^{pos} cells (predominant in mucosa) and Vδ3^{pos} cells, which comprise 0.1% of γδ T cells in peripheral blood (Xiong and Raulet, 2007). This is reviewed in more detail in Section (1.2.2.3).

Dechanet-Merville and colleagues have shown that γδ T cells are considerably expanded (reaching 40% of circulating T cells) following primary CMV infection in CMV-seronegative transplant recipients of kidneys from CMV-seropositive donors (Dechanet

et al., 1999a, Dechanet *et al.*, 1999b). Furthermore, an early γδ T cell reconstitution seems to be associated with improved control of CMV replication, and the expansions were composed of either $V\delta1^{pos}$ or $V\delta3^{pos}$ cells, but not $V\delta2^{pos}$ cells (Lafarge *et al.*, 2001). Similarly, $V\delta2^{neg}$ γδ T cell expansions have also been reported recently in CMV-infected allogeneic stem cell transplant patients and in CMV-infected fetuses (Knight *et al.*, 2010, Vermijlen *et al.*, 2010).

The selective expansion of $V\delta2^{neg}$ γδ T cells ($V\delta1^{pos}$ and $V\delta3^{pos}$) in CMV-infected hosts implies that these γδ T cells are involved in immunity to CMV. Expanded $V\delta2^{neg}$ γδ T cell lines specifically secrete cytokines and demonstrate cytotoxicity after incubation with CMV-infected target cells *in vitro*, but not uninfected targets or targets cells infected with other herpes viruses (Halary *et al.*, 2005). Though the ligand remains undefined, it was shown that $V\delta2^{neg}$ γδ T cells are also expanded in healthy CMV carriers (Pitard *et al.*, 2008).

The association between CMV status and oligoclonal expansions of both CD4+ and/or CD8+ CMV-specific memory T cells in CMV healthy carriers is well established (Karrer *et al.*, 2003, Pourgheysari *et al.*, 2007). Furthermore, this cell accumulation seems to be age related, for instance, CMV specific αβ T cell levels are frequently over 1% of the respective subset in young virus carriers, and often exceed 10% of CD4+/CD8+ T cells in the elderly (Khan *et al.*, 2004, Pourgheysari *et al.*, 2007). The question whether, like CMV-specific CD4+ and CD8+ T-cells, $V\delta2^{neg}$ γδ T cells also expand with age has seemingly not been addressed yet.

4.2 Aims

The persistent nature of CMV infection is thought to drive the accumulation of large CD4+/CD8+ T cell expansions over time. Thus the aim was to test the hypothesis that $V\delta2^{neg}$ γδ T cell expansions occur more frequently in CMV carriers of older age and also to test whether there are similarities between the phenotype and properties of $V\delta2^{neg}$ γδ T cells and αβ T cells in this group.

4.3 Plan of investigation

To achieve these aims, the investigation plan was designed to involve successive steps. Firstly the CMV status of the study cohort was determined by detecting CMV-specific IgG antibody by ELISA. Then the study population was categorized according to age into three groups namely; young, middle aged, and elderly. This was followed by comparing the frequency and absolute number of different γδ T cells subsets between CMV-seropositive and CMV-seronegative subjects and also between different age groups. Furthermore, the $V\delta2^{neg}$ γδ T cell phenotype was determined and compared to that of αβ T cells. Finally, some functional studies of virus recognition by $V\delta2^{neg}$ γδ T cells were also carried out. Figure 4.1 summarises these steps of the investigation.

4.4 Statistical analyses

These were performed with Graphpad Prism software (GraphPad Software Inc.). The Mann Whitney test was applied with 95% confidence intervals to test differences in γδ T cell frequencies between different donor groups. The non-parametric Spearman's rank correlation coefficient was used to assess correlations between different T cell subset frequencies. All P values were two-tailed.

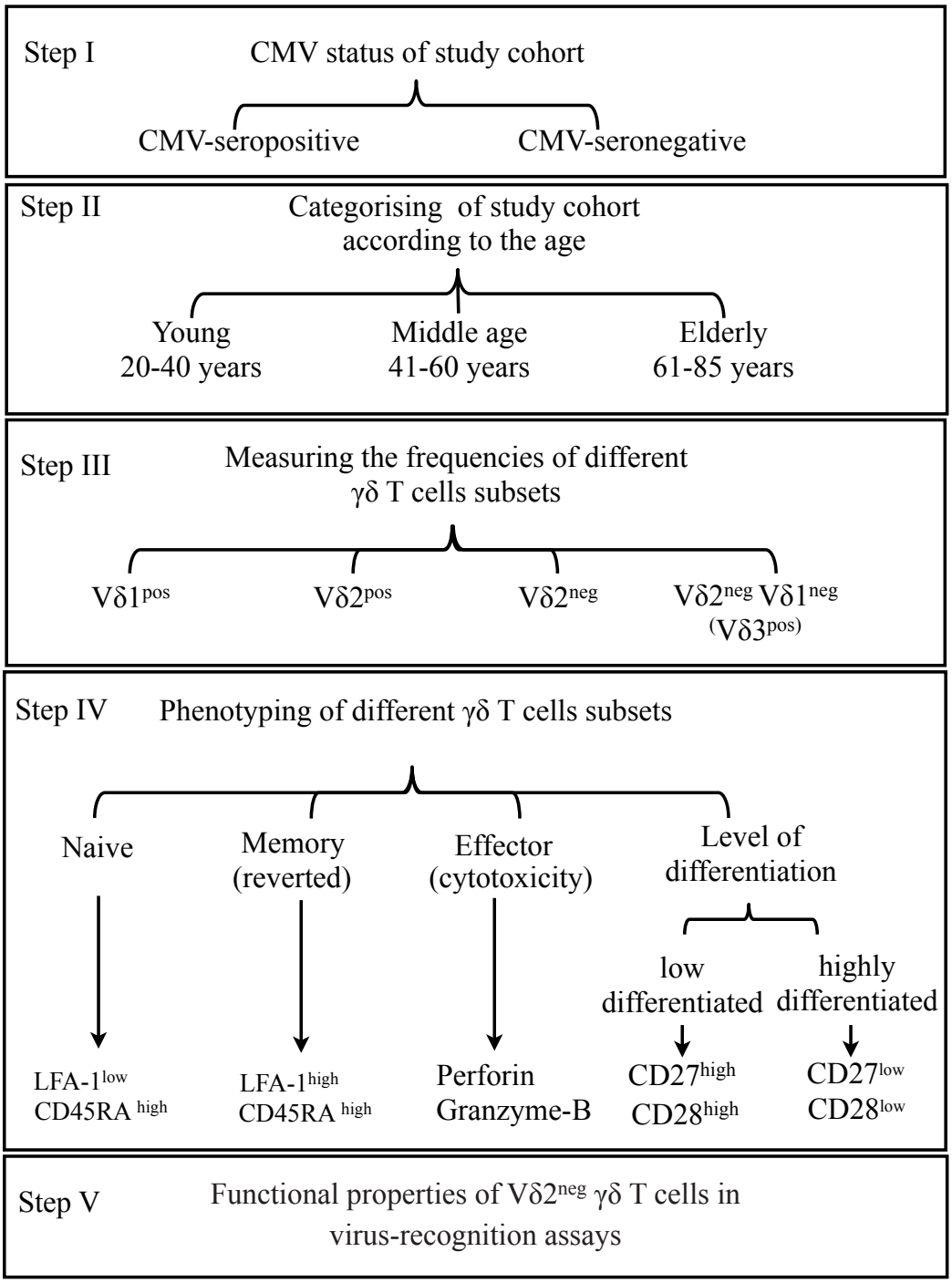


Figure 4.1: Summary of investigation steps

4.5 Results

4.5.1 Study volunteers

255 healthy adult volunteers, aged 20-85 years, and two non-immunocompromised patients diagnosed with symptomatic primary CMV infection, were formally consented to donate blood samples for the study. All subjects were mobile, did not have any cognitive impairment, were not suffering from acute or chronic illness, and were not on medication known to affect the immune system. Ethical approval was obtained from the local Adult Research Ethics Committees. CMV status was determined using plasma samples with a commercial CMV IgG ELISA kit (Biocheck Inc.). Diamedix HSV IgG and VZV IgG kits (Launch Diagnostics) were used for herpes simplex virus (HSV) and varicella zoster virus (VZV) seropositivity, see methodology section 2.3.1. Absolute lymphocyte counts were determined by the Blood Sciences Department at the Royal Liverpool University Hospital NHS Trust. Table 4.1 below summarises the study population characteristics.

Table 4-1: Study cohort characteristics

Age group	CMV-positive	CMV-negative	Total
21-40 years	39	58	97
41-60 years	43	40	83
61+ years	43	32	75
Total	125	130	255

4.5.2 Influence of CMV carriage on γδ T cell subset repertoire

After categorizing of the study cohort into CMV-seropositive and CMV-seronegative, the next step was to identify frequencies of different γδ T cell subsets. Therefore flow cytometric analysis of PBMC utilizing a panel of monoclonal antibodies was carried out. This included staining with anti-panTCRγδ, anti-Vδ1 and anti-Vδ2 monoclonal antibodies to determine the frequency of $V\delta1^{pos}$ γδ T cells, $V\delta2^{pos}$ γδ T cells and $V\delta2^{neg}$ γδ T cells, see Figure 4.2. Furthermore, the frequency of $V\delta3^{pos}$ γδ T cells was estimated (due to lack of an anti-Vδ3 monoclonal antibody) by applying the following formula:

% $V\delta3^{pos}$ γδ T cells = % total $V\delta2^{neg}$ γδ T cells - % $V\delta1^{pos}$ γδ T cells

Overall, the frequency of different γδ T cell subsets showed considerable variation; with some individuals $V\delta1^{pos}$ cells were the major γδ T cell type while in others $V\delta2^{pos}$ cell expansion was observed (see representative examples in Figure 4.2 A and B).

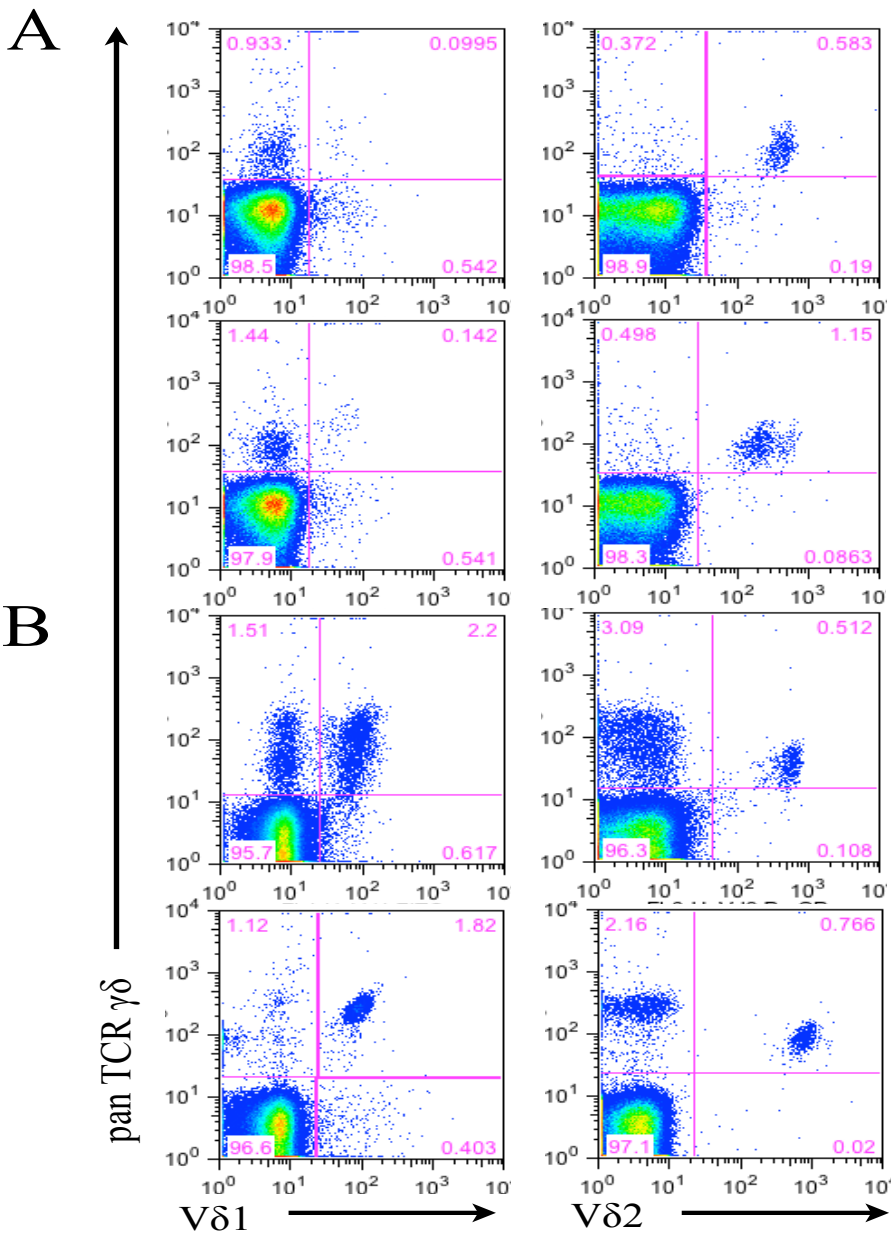


Figure 4.2: γδ T cell subsets in healthy donors

Flow cytometry plots showing PBMC stained with pan-TCRγδ and anti-Vδ1 or anti-Vδ2 monoclonal antibodies from 2 CMV-seronegative (A) and 2 CMV-seropositive healthy donors (B). Values shown in the quadrants of each plot indicate the percentage of lymphocytes staining for each γδ subset.

4.5.3 CMV carriage associated with increase of Vδ2^{neg} γδ T cell frequency that is composed of Vδ1^{pos} and Vδ3^{pos} γδ T cells.

Interestingly, comparison between the total γδ T cell frequency in CMV-seropositive and CMV-seronegative donors was very similar (Figure 4.3A). Nevertheless, Vδ2^{neg} γδ T cells were significantly higher ($p<0.0001$) in CMV-seropositive donors than in CMV-seronegative donors (see Figure 4.3B). This coincided with a slight reduction of Vδ2^{pos} γδ T cells in CMV carriers but this was not statistically significant (Figure 4.3B). This study also confirmed that the Vδ2^{neg} γδ T cell population was composed mainly of Vδ1^{pos} and to a lesser degree Vδ3^{pos} γδ T cells (γδ T cells negative for both Vδ1 and Vδ2). Relative numbers of both types of Vδ2^{neg} γδ T cell was significantly higher (Vδ1: $p<0.0001$ and Vδ3 [Vδ1^{neg} Vδ2^{neg}]: $p=0.0238$) in CMV-seropositive donors than in seronegatives (Figure 4.3C).

However, to confirm that this effect was CMV-associated, other human herpes-viruses, HSV-1/-2, and VZV were tested for. Statistical analysis did not show any significant difference in γδ T cell subsets between seropositive and seronegative donors for these viruses (data not shown), in agreement with work published by others (Pitard *et al.*, 2008).

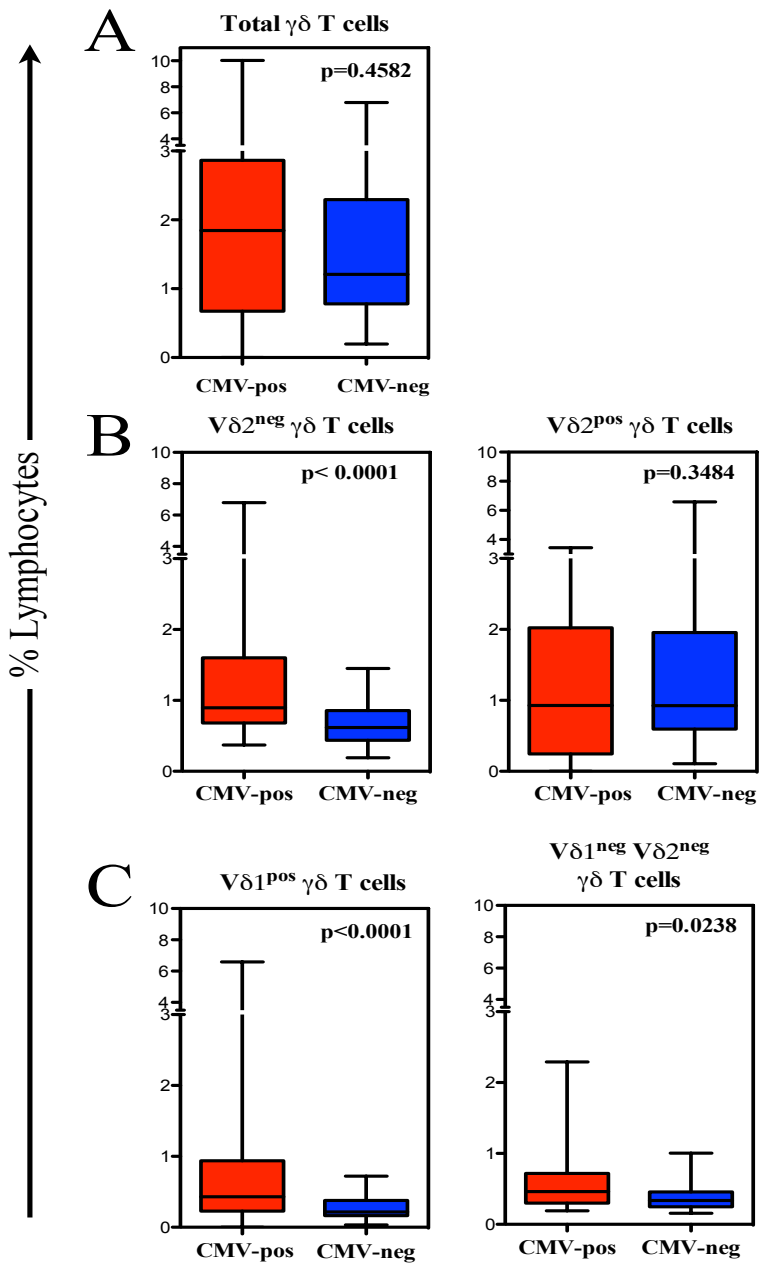


Figure 4.3: CMV carriage characterized by increase in the frequency of Vδ1^{pos} and Vδ3^{pos} γδ T cells.

Charts summarising the staining results from 182 healthy donors are shown for: total γδ T cells (A), Vδ2^{pos} and Vδ2^{neg} γδ T cells (B), and for Vδ1^{pos} and Vδ1^{neg} δ2^{neg} γδ T cells (C). Values on the y-axis indicate the percentage of total T lymphocytes represented by each subset. P values are shown above each plot with 95% confidence intervals applied.

4.5.4 Ageing may also be a factor in driving V2δ^{neg} γδ T cell expansions in CMV-seropositive donors.

To test if Vδ2^{neg} γδ T cell expansions were more evident in older CMV carriers, donors were separated into three age groups: young, 21-40 years old (n=97), middle-aged, 41-60 years old (n=83) and elderly, 61 years or over (n=75). Interestingly, a significant increase in the frequency of Vδ2^{neg} γδ T cells was observed in CMV carriers of all age groups when compared with age-matched CMV-seronegative donors. This increase was also significant in all age groups when expressed as absolute numbers of cells per μl of blood (young; $p=0.009$, middle aged; $p=0.0003$ and elderly donors; $p<0.0001$), see figure 4.4A-C.

In contrast, Vδ2^{pos} γδ T cells were reduced in CMV-seropositive when compared to CMV-seronegative subjects (Figure 4.4A-C), but this did not reach statistical significance. A number of middle-aged and elderly donors had Vδ2^{neg} γδ T cell expansions approaching 10% (or more) of all T cells, with the highest observed frequency at 41% of all T cells in one healthy elderly donor. These were mainly composed of Vδ1^{pos} cells (data not shown). Overall, these findings are very similar to that of increased CMV-specific CD4+ and CD8+ T cells in healthy old-aged virus carriers and suggest that persistent infection results in multiple cell types being expanded even in healthy carriers. Table 4.2 summarizes the influence of age and CMV carriage on γδ T cell subsets.

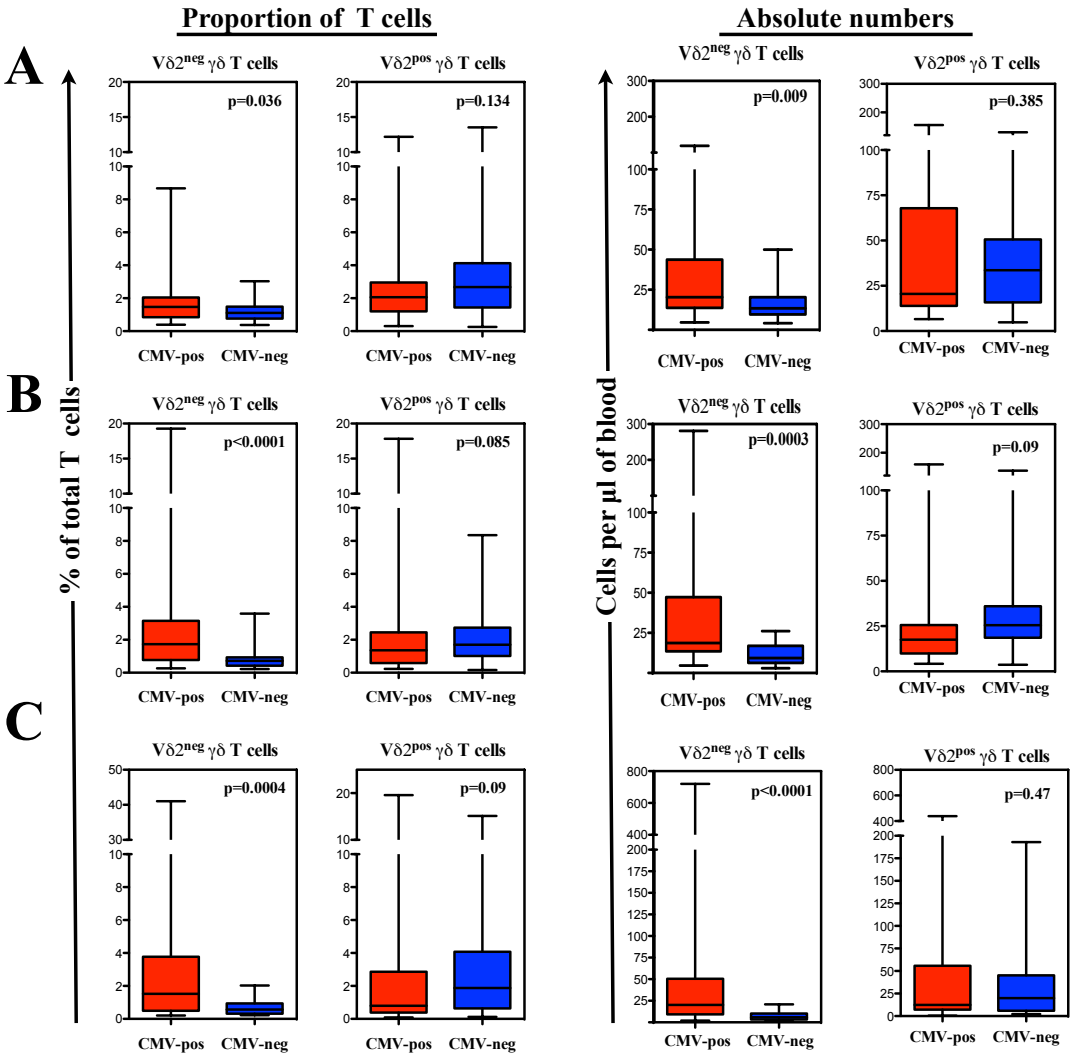


Figure 4.4: The effects of ageing and CMV carriage on γδ T cell subsets

Charts summarising the frequencies of circulating Vδ2^{pos} and Vδ2^{neg} γδ T cells after inclusion of elderly donors and separation into age groups. Young; 21-40yrs (A), middle age; 41-60yrs (B) and elderly 61yrs and over (C). Values on the y axis indicate the percentage of the total T cell repertoire (histograms panels on the left side) and absolute numbers of cells per µl of blood (histograms panels on the right side). All data were derived from a single blood sample.

Table: 4-2 Summary of the influence of ageing and CMV carriage on γδ T cell subsets

Age group	T cell Subset	CMV-pos	CMV-neg	P value (Mann-Whitney)
21-40yrs		(N=39)	(N=58)	
	$V\delta2^{neg}$	2.04% +/- 0.3 (29.71+/-5.75)	1.21% +/- 0.08 (14.58+/-1.5)	0.036 (0.009)
	$V\delta2^{pos}$	2.62% +/- 0.37 (35.5+/-6.4)	3.37% +/- 0.38 (39.5+/-4.7)	0.134 (0.385)
41-60yrs		(N=43)	(N=40)	
	$V\delta2^{neg}$	2.44% +/- 0.46 (40.14+/-9.87)	0.85% +/- 0.1 (11.42+/-1.32)	<0.001 (0.0004)
	$V\delta2^{pos}$	2.17% +/- 0.44 (29.62+/-5.9)	2.44% +/- 0.32 (34.8+/-5.1)	0.085 (0.09)
60+yrs		(N=43)	(N=32)	
	$V\delta2^{neg}$	3.67% +/- 1.03 (58.16+/-25.66)	0.7% +/- 0.09 (7.01+/-1.09)	0.0004 (<0.0001)
	$V\delta2^{pos}$	2.06 % +/- 0.5 (44.1+/-13.8)	3.07% +/- 0.64 (43.7+/-8.9)	0.09 (0.472)

Note, values denote means and standard error for each subset as a percentage of total T cells and, in brackets, absolute numbers per μl of blood.

4.5.5 Identification of naive and memory γδ T-cell subsets

Having observed the differences in γδ T cell subset repertoire by CMV carriage and age, we asked whether this population consists of naive or memory cells. To answer this question the expression of LFA-1 and CD45RA by $V\delta2^{pos}$ and $V\delta2^{neg}$ γδ T cells was measured by flow cytometry. Accordingly three major cell subsets were identified namely; *Naive* (LFA-1^{low} $CD45RA^{high}$), *Memory* "normal" also called T_{EM} ($LFA1^{high}$ $CD45RA^{low}$), *Memory* **"**revertant" (LFA-1^{high} $CD45RA^{high}$) also denoted as T_{EMRA}. Figure 4.5 demonstrates gating steps for identifying these cell subsets.

Representative examples of these cells subsets based on sub-gating on $V\delta2^{pos}$ or $V\delta2^{neg}$ γδ T cells in CMV-seropositive and CMV-seronegative donors are shown in Figure 4.6 (A and B). While $V\delta2^{pos}$ cells were overwhelmingly $CD45RA^{low}$ memory cells in both CMV-seropositive and CMV-seronegative donors (Fig. 4.6B), $V\delta2^{neg}$ cells showed a distinct naive/memory profile (Fig. 4.6A), which appeared to be linked to CMV status. For instance, in CMV-seropositive donors the $V\delta2^{neg}$ γδ T cells subset was skewed towards $CD45RA^{high}$ revertant memory cells, this coincided with reduced frequency of naive cells. Changes in the phenotype profile of γδ T cell subsets according to the CMV status are summarized in Table 4.3.

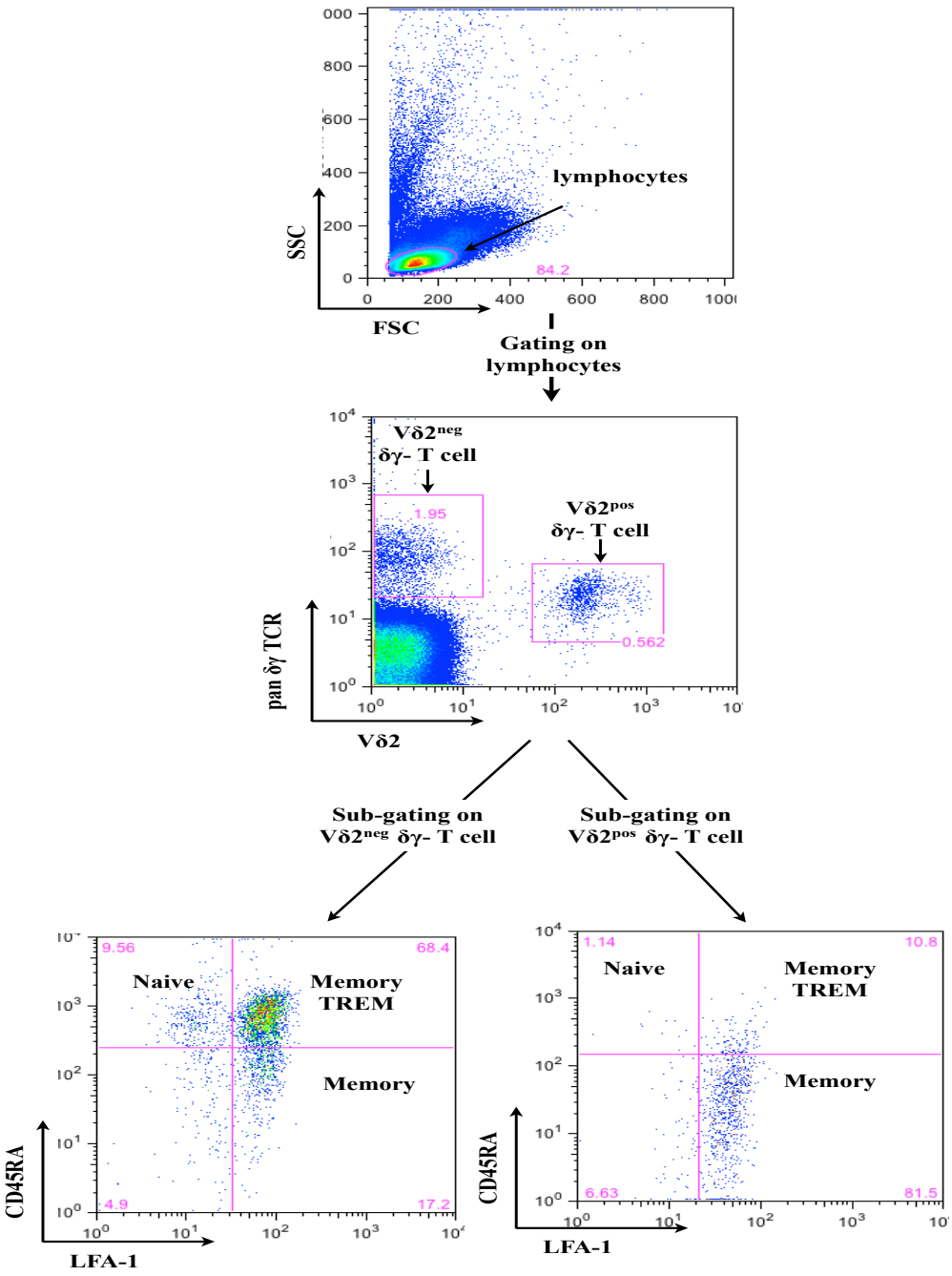


Figure 4.5: Steps for identifying phenotypic profiles for Vδ2^{pos} and Vδ2^{neg} γδ T-cell subsets.

PBMC were stained with anti-TCRγδ, Vδ2, LFA1 and CD45RA monoclonal antibodies. Flow cytometry plots show LFA-1 vs CD45RA staining of Vδ2^{pos} and Vδ2^{neg} γδ T-cell subsets. Therefore, each cell subset can be categorized into *Naive* (LFA-1^{low} CD45RA^{high}), *Memory* "normal" (LFA-1^{high} CD45RA^{low}), *Memory* "revertant" (LFA-1^{high} CD45RA^{high}).

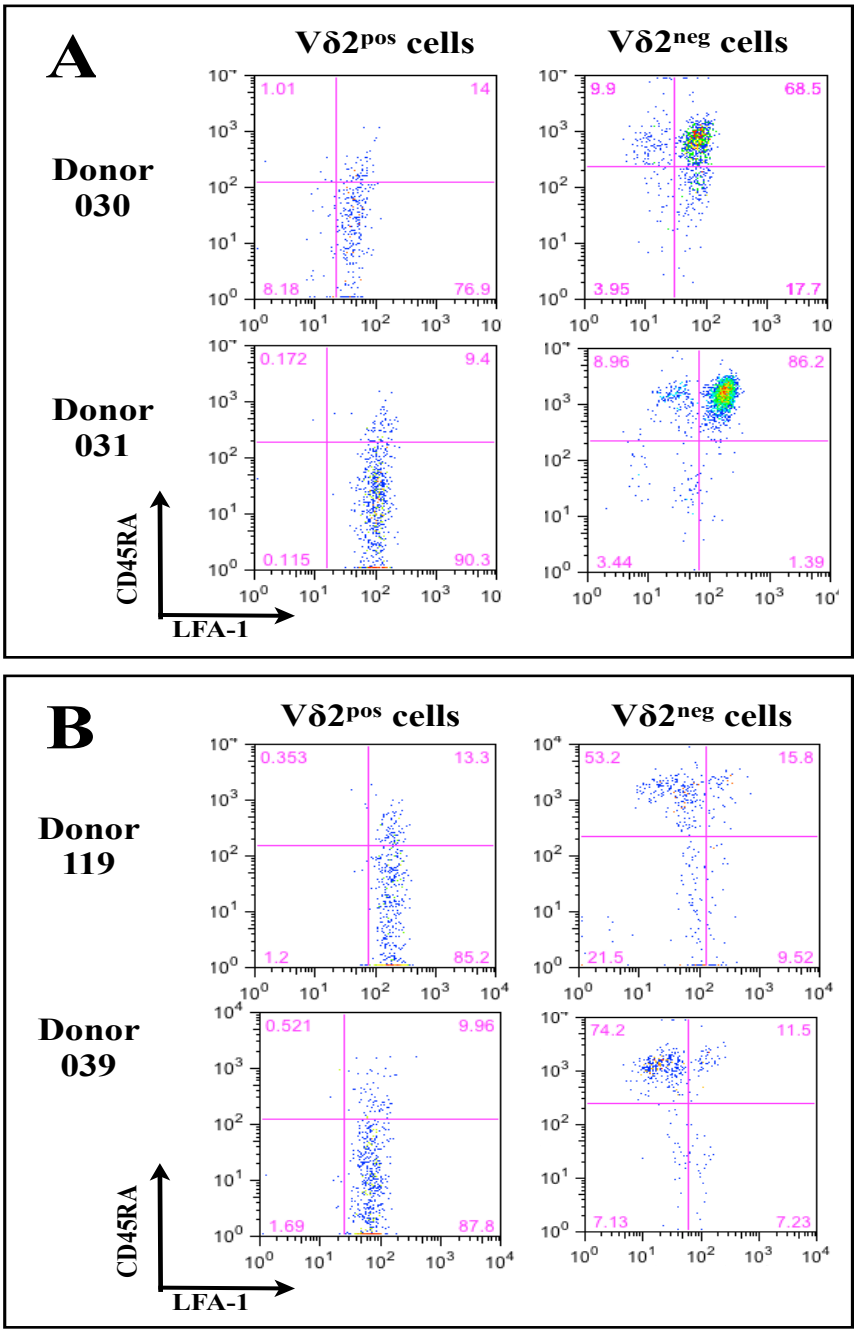


Figure 4.6: Influence of CMV carriage on naive and memory cell composition of γδ T cell subsets

Plots are illustrative example of γδ T cell subsets phenotyped according to expression of LFA-1 vs. CD45RA from two CMV-seropositive donors (A) and two CMV- seronegative donors (B). Values indicate percentage of gated cells in each quadrant.

Table 4-3 Summary of effects of CMV status on phenotypic profiles of γδ T cell subsets

Phenotype	T cell Subset	CMV-neg (n=50)	CMV-pos (n=50)	P value (Mann-Whitney)
Naive **(LFA-1^{low} CD45RA^{high})**				
	Vδ2^{neg}	3.08 (1.51-7.39)	3.93 (1.75-7.55)	0.5999 (369.0)
	Vδ2^{pos}	0.32 (0.17-0.48)	0.28 (0.15-0.54)	0.5913 (321.0)
Memory **"normal" (LFA-1^{high} CD45RA^{low})**				
	Vδ2^{neg}	1.47 (0.96-3.01)	1.63 (0.81-3.62)	0.8800 (392.5)
	Vδ2^{pos}	21.39 (10.29-44.55)	14.31 (9.18-20.29)	0.0561 (243.0)
Memory **"revertant" (LFA-1^{high} CD45RA^{high})**				
	Vδ2^{neg}	2.72 (1.43-6.31)	6.60 (2.624-18.06)	0.0151 (434.5)
	Vδ2^{pos}	5.28 (2.07-11.60)	2.75 (0.90-5.80)	0.0420 (236.0)

Values denote median with lower and upper percentile (in brackets) for each subset as an absolute numbers per μl of blood.

4.5.6 Age *per se* has its influence on the naive and memory Vδ2^{neg} γδ T cell subset repertoire

Having observed substantial differences in frequency and also in absolute number of the memory revertant Vδ2^{neg} γδ T cell subset between CMV-seropositive and CMV seronegative donors, the aim was to find out whether age *per se* has a role in configuring the phenotype of Vδ2^{neg} γδ T cell, independent of CMV status.

Therefore, the first step was to compare absolute numbers of both naïve and revertant memory Vδ2^{neg} γδ T cells between different age group, independent of CMV status. This analysis showed that there was a significant decrease in absolute numbers of both naïve and revertant memory cells in the elderly (Figure. 4.7A and B) when compared with middle-aged and young donors (both $p<0.05$).

The second step was to investigate the combined influences of both CMV carriage and age. As can be seen in Figure 4.7C CMV positivity was associated with reduced naive Vδ2^{neg} cells in each group, but this only reached statistical significance in the elderly ($p=0.01$). Meanwhile, there was a significant increase in the absolute count of revertant memory cells in CMV-positive compared to CMV-negative donors in all age groups (Figure 4.7D).

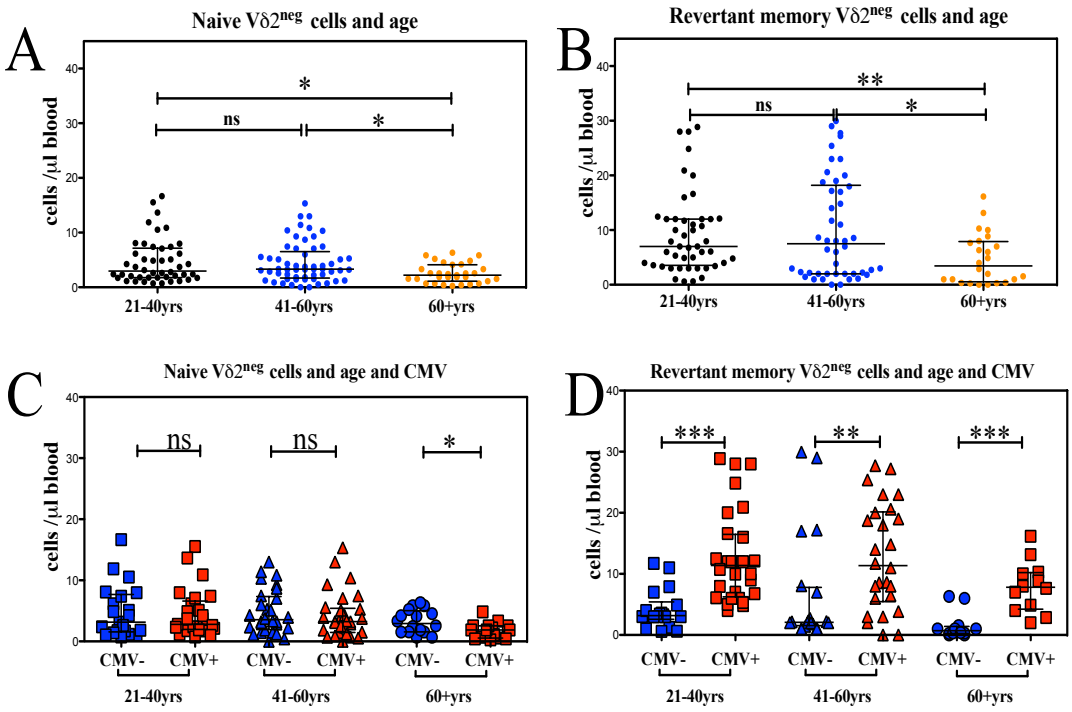


Figure 4.7: Influences of age and CMV status on naïve and revertant memory Vδ2^{neg} γδ T cells.

Study cohort was categorized according to age into three groups; young, middle aged, and elderly. Absolute count of both naive (A) and revertant memory (B) Vδ2^{neg} γδ T cells were compared between age groups, independent of CMV status. Then each age group was further subdivided according CMV status into CMV-seropositive and CMV–seronegative (marked as CMV+ and CMV- on x axis respectively) donors (chart C & D). Error bars indicate median with upper and lower interquartile, meanwhile asterisks denote P value (ns= not significant, *= $p<0.05$, **= $p<0.001$, and ***= $p<0.0001$).

4.5.7 Comparative analysis of Vδ2^{neg} γδ T cell subset with virus-specific CD4+and CD8+ T cells

Although memory Vδ2^{neg} γδ T cells were higher in CMV-seropositive groups, there was considerable inter-individual variation within all age groups. It was questioned whether this variation was due to differences in frequencies of CMV-specific CD4+ and CMV-specific CD8+ T cells; both parameters also varying considerably between individuals in each group. CD4+ T cell frequency was based on IFNγ responses against CMV lysate and CD8+ T cell responses were based on responses against a peptide cocktail representing 6 immunodominant antigens (IE-1, IE-2, pp65, pp50, gB, pp150), which would cover 90% of responders. It is acknowledged that this would not give the complete CMV-specific T cell response, which could involve over 100 viral antigens (Sylwester *et al.*, 2005), however this would be impractical to measure in a large cohort study such as this. The results (Figure 4.8) showed that frequencies of memory Vδ2^{neg} γδ T cells did not correlate with the CD8+ T cell response (r^2=0.034; p=0.847) or CD4+ T cell response (r^2= 0.102; p=0.559). Some individuals had large Vδ2^{neg} γδ memory T cell expansions and weak CMV-specific CD8+/CD4+ T cell responses; some had strong CMV-specific CD8+/CD4+ T cell responses and low frequencies of Vδ2^{neg} memory cells, and some with high levels of all subsets.

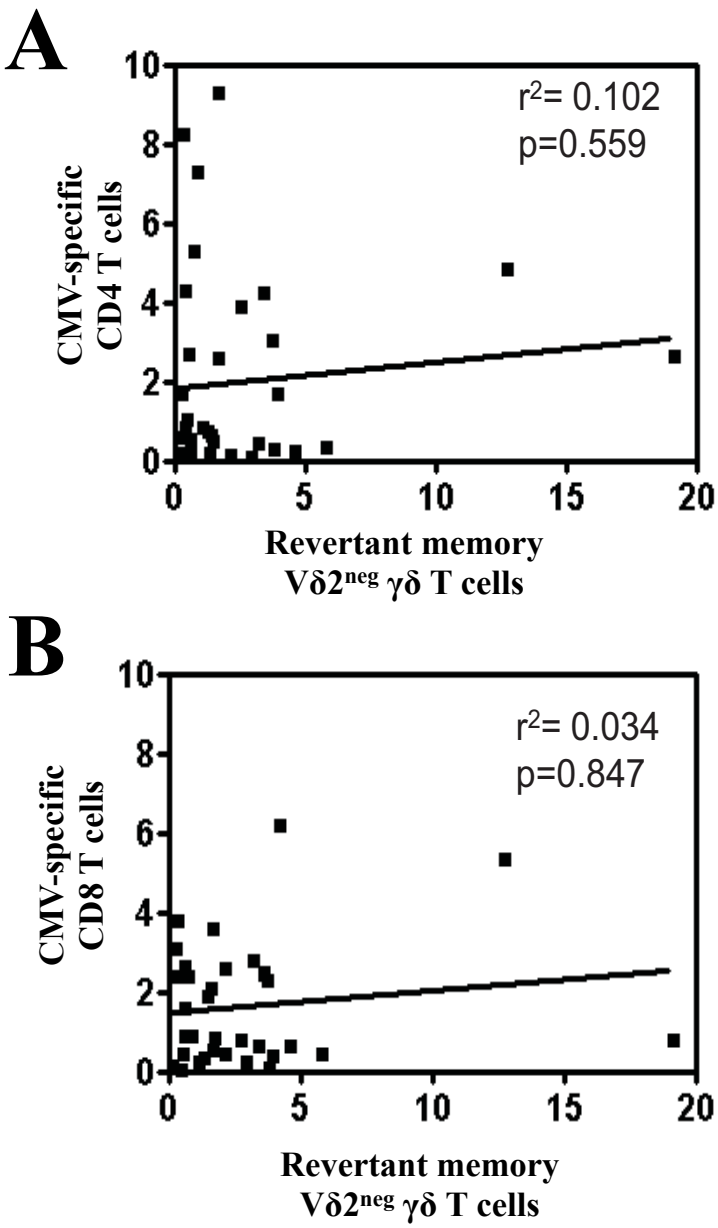


Figure 4.8: Relation between Vδ2^{neg} γδ memory T cell and CMV-specific αβ T cell frequencies in healthy donors

Charts show the correlation between Vδ2^{neg} γδ memory T-cells and CMV-specific CD8+ T-cells (A) and CMV-specific CD4+ T-cells (B) in CMV-seropositive donors. Values on each axis denote the percentage of each cell type as a percentage of the total T-cell repertoire. Significance (p) values at 95% confidence intervals are shown for each set of data. Correlations were determined using the non-parametric Spearman Rank test.

4.5.8 Phenotypic comparison of γδ T cell subsets and CMV-specific αβ T cells

The above described similarity in phenotype between $V\delta2^{neg}$ γδ T cells and CMV-specific CD8+ T cells led to further comparative studies. Previous studies have shown that CMV drives CD4+ and CD8+ T cells towards a highly differentiated Tem ($CD45RA^{low}$) and T_{EMRA} ($CD45RA^{high}$) phenotype (Appay *et al.*, 2002, Pourgheysari *et al.*, 2007). Since $V\delta2^{neg}$ γδ T cells were increased in CMV carriers and, in common with CMV-specific CD4+ and CD8+ T cells, also appeared to increase with age, it was hypothesized that these γδ T cells subset would also be driven towards a highly differentiated T_{EM}/ T_{EMRA} phenotype. Therefore the expression of CD27 vs. CD28 and perforin vs. granzyme B by γδ T cell subsets and CMV-specific αβ T cells in CMV-seropositive and CMV-seronegative donors were compared.

Figure 4.9A shows a representative example of $V\delta2^{neg}$ γδ T cells and $V\delta2^{pos}$ γδ T cells also CMV-specific CD4+ T cells and CMV-specific CD8+ T cells (Fig. 4.9B), analysed within a selected individual CMV-seropositive donor, and the phenotype of $V\delta2^{neg}$ γδ T cells and $V\delta2^{pos}$ γδ T cells in a typical CMV-seronegative donor (Fig. 4.8C). The complete data from CMV-seropositive and CMV-seronegative donors is shown in Figure 4.10.

It is clear that $V\delta2^{neg}$ γδ T cells show remarkable similarity to CMV-specific CD8+ T cells in particular and CMV-specific CD4+ T cells to a lesser degree, but not to $V\delta2^{pos}$ γδ T cells. $V\delta2^{neg}$ γδ T cells in CMV-seropositive donors were composed of greater numbers of late memory ($CD27^{low}CD28^{low}$) cells. In addition $V\delta2^{neg}$ γδ T cells contained higher levels of intracellular perforin and granzyme B than in CMV-seronegative donors,

which probably indicates greater differentiation and functionality of $V\delta2^{neg}$ γδ T cells in terms of cytotoxicity.

In contrast, $V\delta2^{neg}$ γδ T cells in CMV-seronegative donors (Fig. 4.9C) were mostly composed of T_{EM} cells with less differentiated $CD27^{high}CD28^{high}$ phenotype. Further contrast was displayed by $V\delta2^{pos}$ γδ T cells, which were almost exclusively $CD27^{high}CD28^{high}$, with considerable heterogeneity in perforin and granzyme B expression in both CMV-seropositive and seronegative donors. This indicates that CMV carriage leads to a profound change in phenotype of $V\delta2^{neg}$ γδ T cells but not $V\delta2^{pos}$ γδ T cells. See Figure 4.10 for complete data set analysis.

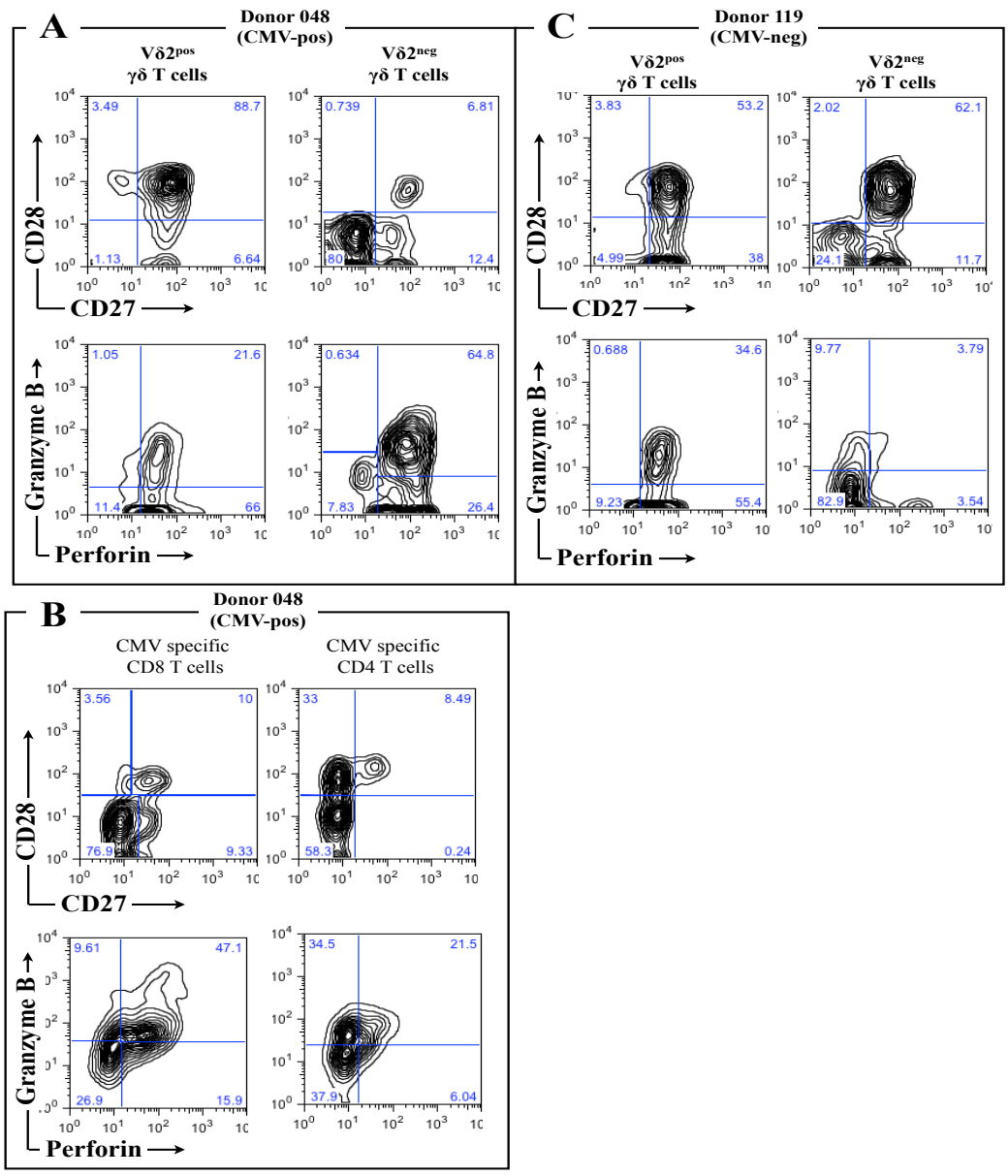


Figure 4.9: Phenotypic comparison of γδ T cell subsets and CMV-specific αβ T cells

Flow cytometry plots of CD27 vs. CD28 and perforin vs. granzyme B staining of gated γδ T cells from PBMC of one CMV-seropositive (A&B) and one CMV-seronegative donor (C). The events shown are gated on T cell subsets indicated above each column of plots. For CMV-specific αβ T cells, events are gated on CMV tetramer (A1-VTE) binding CD8+ T cells and ex vivo interferon-gamma producing CD4+ T cells after 6hr stimulation with CMV lysate at 37°C.

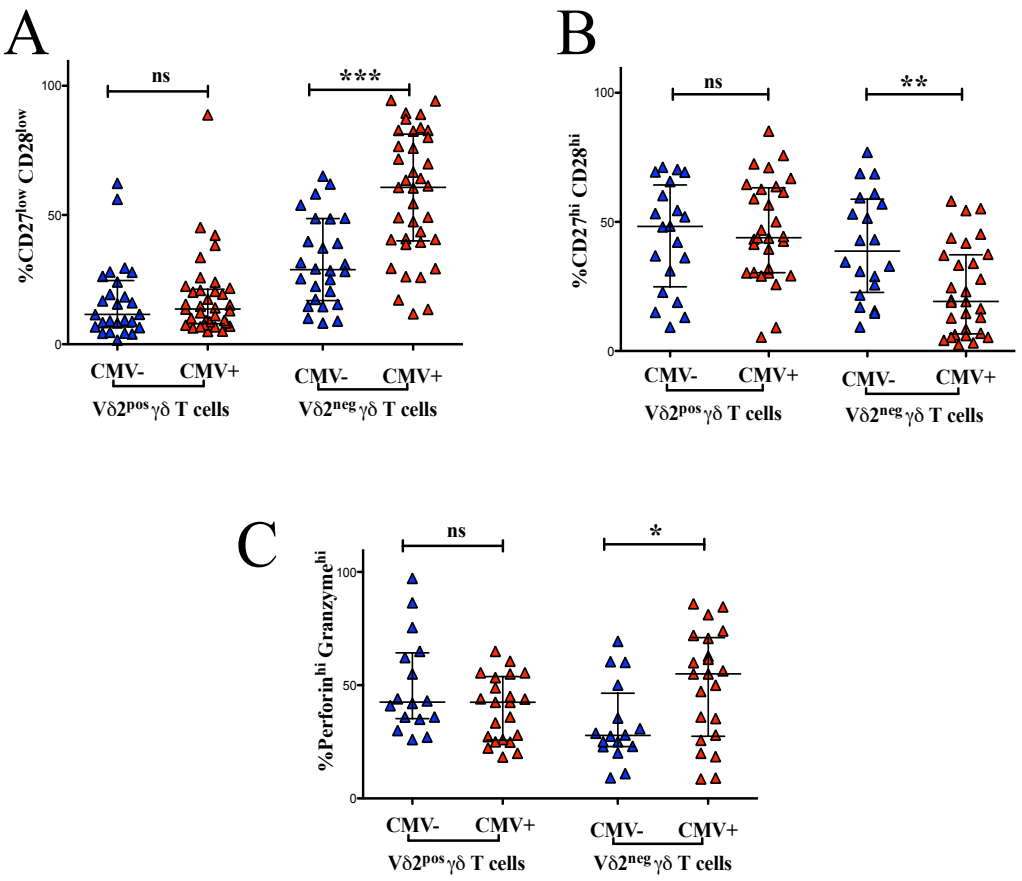


Figure 4.10: Level of differentiation and cytotoxicity ability of γδ T cell subsets.

It summarizes differences in the level of differentiation (A&B) and cytotoxic capacity (C) of $V\delta2^{neg}$ γδ T cells and $V\delta2^{pos}$γδ T cells in CM-seropositive (red triangles) and CMV-seronegative (blue triangles). Error bars indicate median with interquartile range, while asterisks denote P value (*= $p<0.05$, **= $p<0.001$, and ***= $p<0.0001$).

4.5.9 Dynamics of Vδ2^{neg}γδ T-cells in healthy donors over time

Levels of herpesvirus-specific αβ T cells are reported to fluctuate over time (Crough *et al.*, 2005). Thus it was interesting to learn if Vδ2^{neg} γδ T cells displayed this behaviour by performing longitudinal analysis on a random selection of 6 CMV-seropositive and 6 CMV-seronegative donors and two cases of primary CMV-infectious mononucleosis (IM) infection. Vδ2^{neg} cell numbers varied modestly in healthy donors over time (see Figure 4.11A), mean 29.2% variation in CMV-seropositive and mean 35.7% in CMV-seronegative donors, while CMV-IM patients showed a more dramatic reduction in absolute number of Vδ2^{neg} γδ T cells in samples collected between 1 week post-diagnosis and subsequent time points (Figure 4.11B). All healthy donors showed very stable phenotypes at each time point, but in CMV-IM Vδ2^{neg} γδ T cells were initially composed of normal memory (T_{EM}) and revertant memory (T_{EMRA}) cells, and both CD27^{high}CD28^{low} and CD27^{low}CD28^{low} cells during the early phase of infection. However, after 2 years Vδ2^{neg} γδ T cells had shifted almost exclusively to the T_{EMRA} phenotype with a concomitant shift to highly differentiated CD27^{low}CD28^{low} cells (Fig. 4.12). This change was more dramatic than those observed in healthy donors. In contrast, Vδ2^{pos} γδ T cells were composed mainly of less differentiated CD27^{high}CD28^{high} CD45RA^{neg} T_{EM} cells both during acute infection and 3 years later (data not shown).

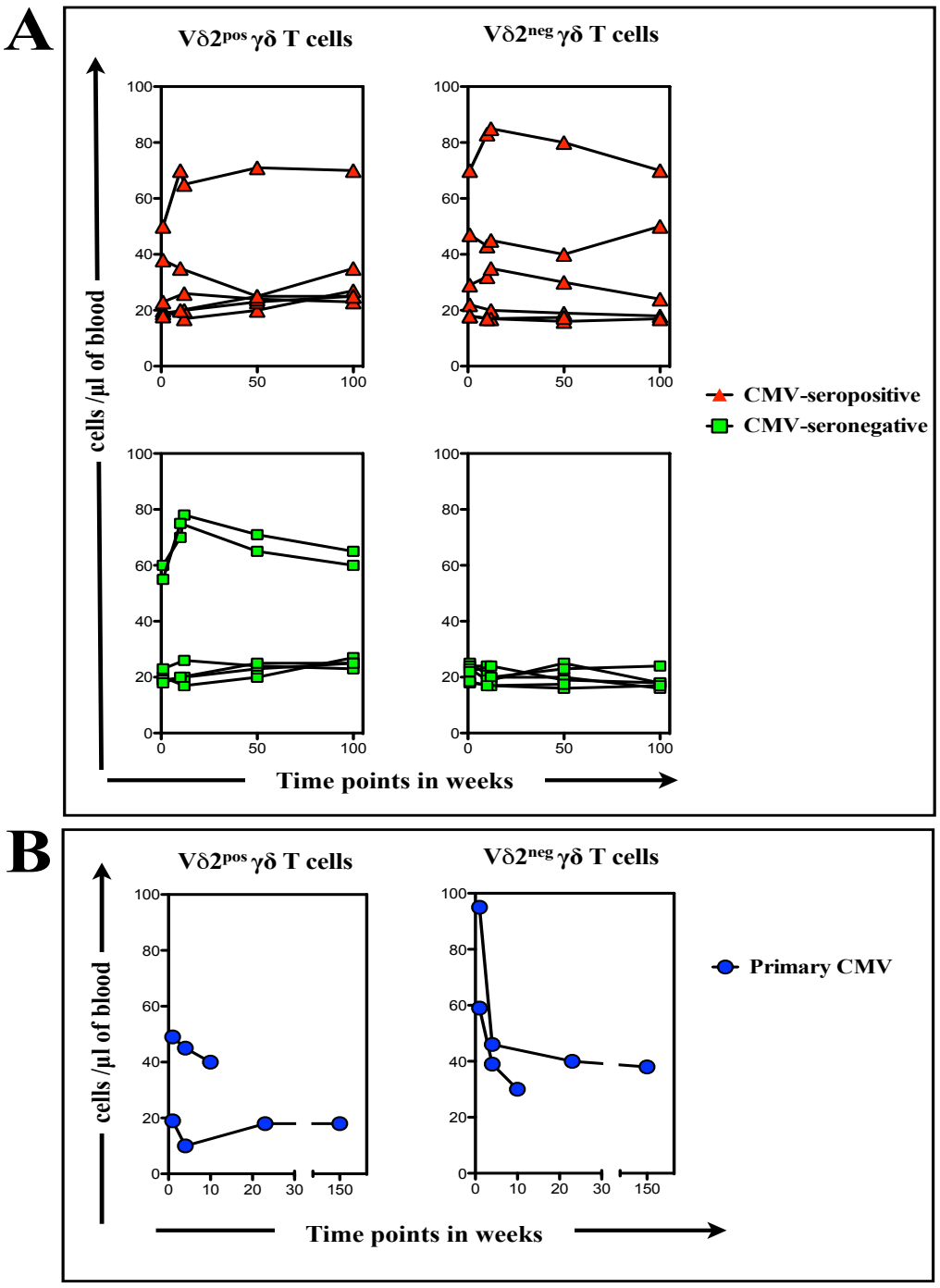


Figure 4.11: Dynamics of Vδ2^{neg}γδ T-cells in healthy donors over time

Vδ2^{pos} and Vδ2^{neg} γδ T -cell numbers were measured in a longitudinal analysis of 6 CMV-seropositive (top charts) and 6 CMV-seronegative healthy donors (bottom charts) (A), and also in 2 immunocompetent subjects diagnosed with primary CMV infection (B).

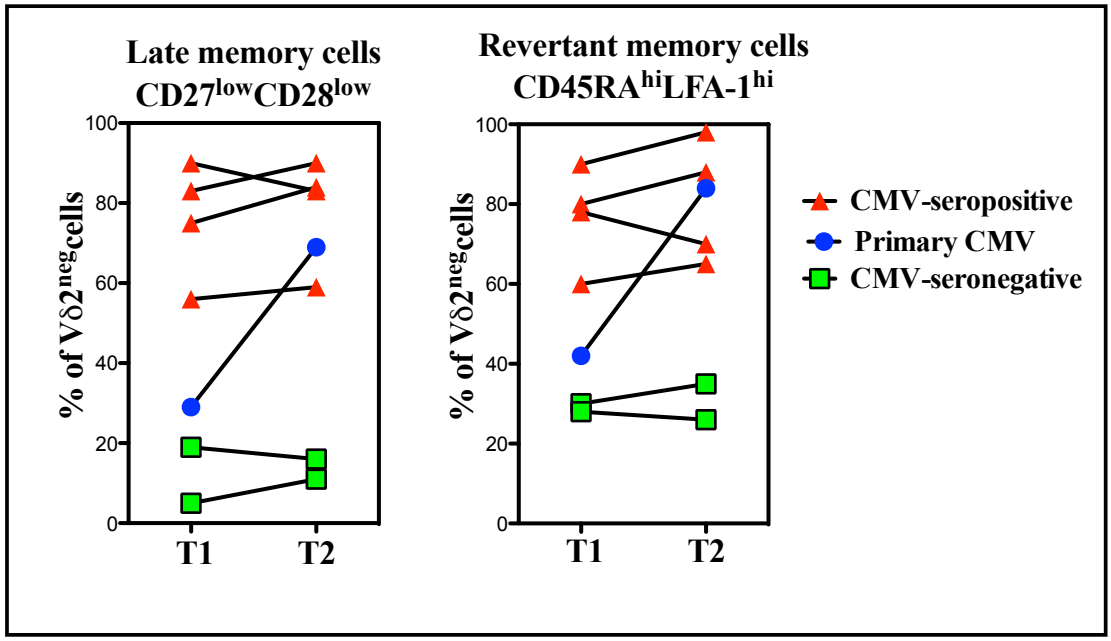


Figure 4.12: Dynamic changes in memory and T_{EMRA} $V\delta2^{neg}$ γδ T cells

Phenotypic changes in late memory and T_{EMRA} cells were determined after a 12-month interval in 6 healthy donors (CMV-pos; red triangles, CMV-neg; green quadrangles) and 1 of the primary CMV patients (blue circle) (C). T1 represents 1 week post-diagnosis and T2 denotes 2 years post-diagnosis for the primary CMV samples. Values shown indicate the percentage of $V\delta2^{neg}$ γδ T cells expressing the given phenotype.

4.5.10 Functional properties of Vδ2^{neg} γδ T cells in virus-recognition assays

4.5.10.1 In vitro generation of γδ T cell lines

To confirm that Vδ2^{neg} γδ T cells had CMV-specific reactivity, γδ T cell lines were generated *in vitro*. Briefly, this consisted of two steps; the first step was enriching γδ T cells of two selected donors, who were characterized by high frequencies of Vδ2^{neg} γδ T cells. One of these donors was CMV-seropositive and the other CMV-seronegative. Here, PBMC were depleted of monocytes by plastic adherence and of Vδ2^{pos} γδ T cells, αβ T cells, B cells and erythrocytes by magnetic selection using a special kit that contains a mouse monoclonal antibody cocktail specific for human Vδ2, TCRαβ, CD19, CD14. For more details see section 2.5.1 and 2.5.2.

The second step was to establish γδ T cell lines by co-culture of enriched γδ T cells with irradiated PHA-activated allogeneic PBMC as feeder cells and stimulation with anti-CD3 and recombinant interleukin 2 (rIL-2). Cultures were enriched further by depletion of αβ T cells prior to re-stimulation with feeder cells (after 3 to 4 weeks) followed by maintenance with media containing rIL-2. The absence of αβ T cells was confirmed by flow cytometry. As controls CMV-specific CD8+ T cell lines from CMV-seropositive donors were generated after stimulation with the cocktail of CMV-encoded peptide epitopes. Figure 4.13 demonstrates the steps of the functional assay.

4.5.10.2 Virus-recognition assays

The outcomes of the previous step (4.5.10.1) was four cell lines namely; two CD8+ T cell lines (one from CMV-seropositive and one from CMV-seronegative), and similarly two $V\delta2^{neg}$ γδ T cells lines. Aiming to test their response to CMV each cell lines was co-cultured either with uninfected human fetal foreskin fibroblasts (HFFF) or with HFFF that had been infected for 3-4 days with the AD169 or Towne strain of CMV (at a multiplicity of infection of 1:1) see section 2.5.4.

Furthermore, to investigate if the responses of each cell line were through TCR receptors several blocking steps were performed. Herein, sets of T cells lines had been initially pre-incubated with anti-TCRαβ, anti-TCRVδ1, anti-TCRVδ2 or mouse isotype control monoclonal antibodies before they were co-incubated with mock and CMV-infected HFFF.

In addition, aiming to test the ex-vivo effector function of $V\delta2^{neg}$ γδ T cells, PBMC from a CMV-seropositive was freshly isolated. A portion of these PBMC was used to test $V\delta2^{neg}$ γδ T cells enriched PBMC while the rest (unfractionated PBMC) was used to assess the αβ T cell response (as control). The schematic in Figure 4.13 summarize these steps of CMV-recognition assay.

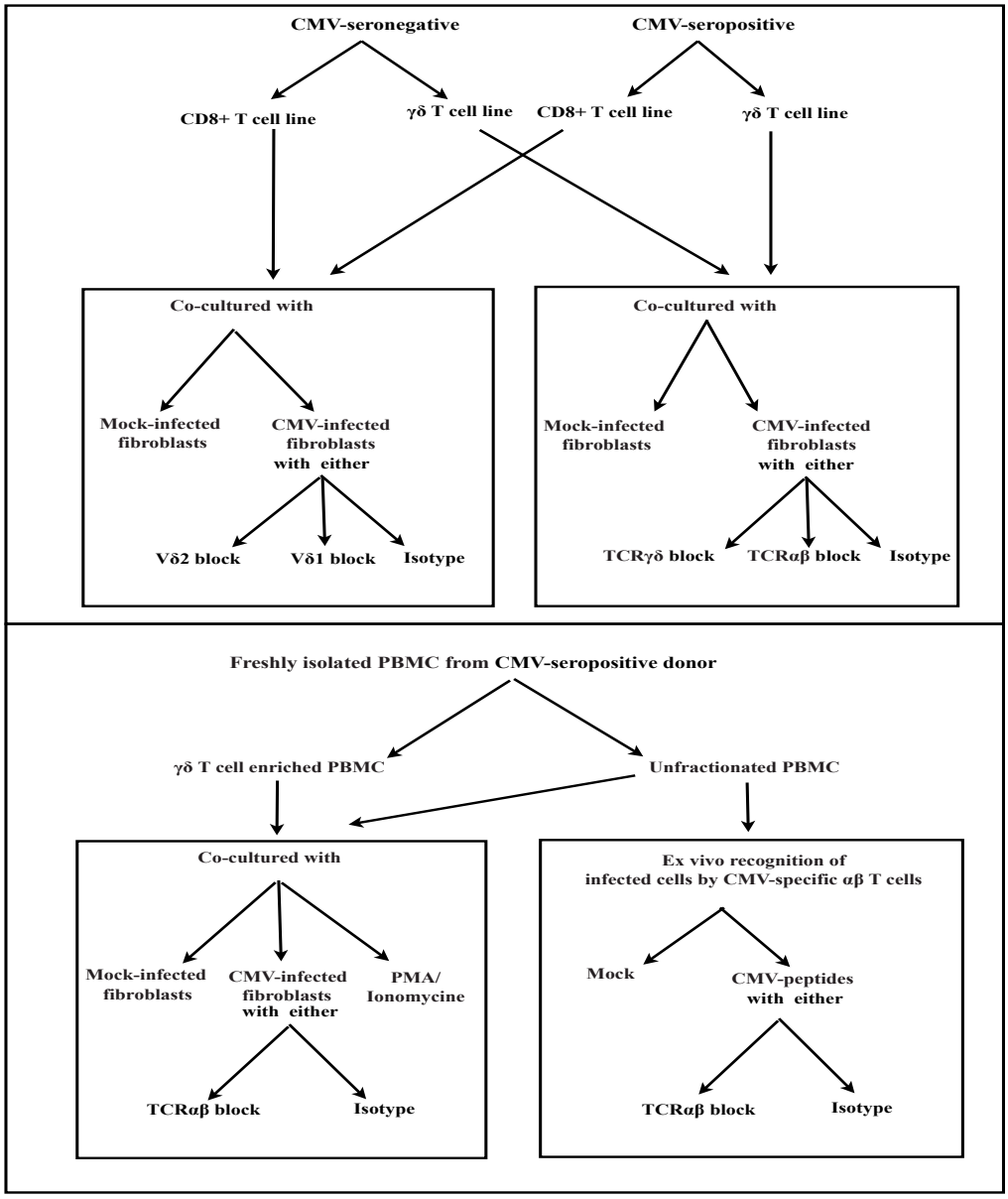


Figure 4.13: Flow charts of functional assay

This is to demonstrate the general scheme for determination of functional properties of Vδ2^{neg} γδ T cells in virus-recognition assays. Basically, Vδ2^{neg} γδ T cell and CD8+ T cell lines were generated from CMV-seropositive and CMV-seronegative healthy donors. This followed by co-incubated them with either CMV-infected or mock-infected HFFF in presences and absence of different blocking monoclonal antibodies (anti-TCRαβ, anti-TCR γδ, anti-TCRVδ1, anti-TCRVδ2 or mouse isotype). Finally, the *ex-vivo* effector function of Vδ2neg γδ T cell was investigated in Vδ2^{neg} γδ T cells enriched PBMC and compared to αβ T cell response (unfractionated PBMC).

Results showed that T cell lines from both sets of donors (CMV-seropositive and CMV-seronegative) could produce cytokines after co-incubation with CMV-infected targets (using AD169 or Towne strains) but not against mock-infected targets (Figure 4.14A-B), as efficiently as CMV-specific CD8+ T cells (Figure 4.14C). This recognition could be blocked, either partially or completely, using the anti-Vδ1 monoclonal antibody but not with the anti-Vδ2 monoclonal antibody. This confirmed that $V\delta2^{neg}$ γδ T cells in these donors were indeed reactive against CMV, with $V\delta1^{pos}$ γδ T cells being a major component of this recognition. We also tested freshly isolated $V\delta2^{neg}$ γδ T cells, assumed to contain effector memory cells that were reactive against CMV-infected cells, for their ability to perform effector functions in *ex vivo* T cell assays. Figure 4.14E shows that fresh $V\delta2^{neg}$ γδ T cells do not produce IFNγ after co-incubation with CMV-infected target cells. This was also the case when TNFα production was measured (data not shown), suggesting that $V\delta2^{neg}$ γδ T cells do not possess immediate effector function to the same degree as CMV-specific αβ T cells in our assay system. It was possible that the extremely efficient recognition of infected targets by virus-specific CD4+ and CD8+ T cells (Figure 4.14G-H) masked the true potential of the γδ T cell response. However this did not appear to be the case as antibody blocking or depletion of CD4+ and CD8+ T cells had no enhancing effect on the γδ T cells in the *ex vivo* assay (Figure 4.14F).

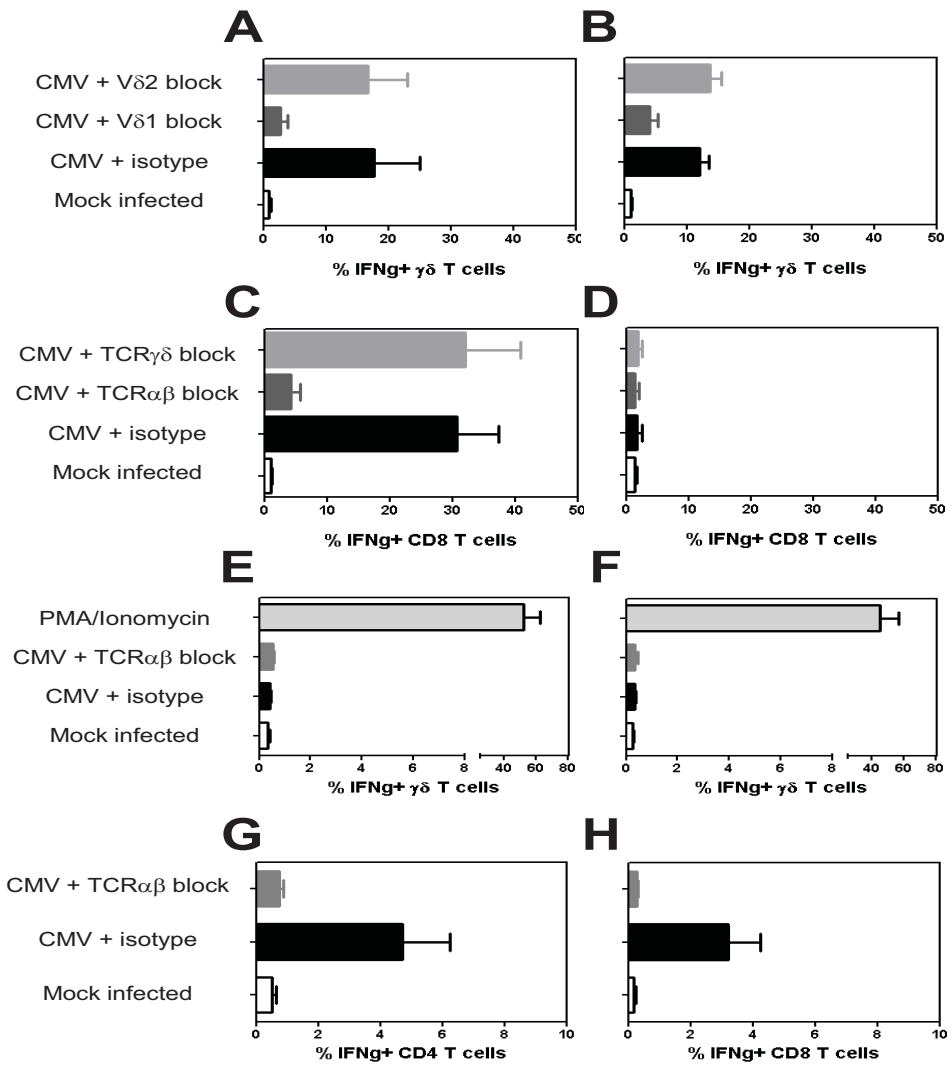


Figure 4.14: Recognition of virus-infected target cells by Vδ2^{neg} γδ T cells.

In vitro OKT3 expanded γδ T cell lines from a CMV-seropositive donor (A), γδ T cell lines from a CMV-seronegative donor (B) and CD8+ T cell lines stimulated with CMV-peptides from a CMV-seropositive donor (C) and CMV-seronegative donor (D) were tested for the ability to recognise CMV-infected (AD169 strain) human fibroblasts. Partially class I HLA matched fibroblasts were used to allow for CD8+ T cell recognition as control effectors. Freshly isolated PBMC were also used for testing immediate effector function of γδ T cells from unfractionated PBMC of CMV positive donor (E) and γδ T cell enriched PBMC (CMV positive)(F).Ex vivo recognition of infected cells by CMV-specific CD4+ T cells (G) and CMV-specific CD8+ T cells (H) in PBMC were also tested. Data is pooled from 3 independent experiments showing IFNγ producing cells detected by flow cytometry. Values on y-axes indicate IFNγ production as the percentage of each T cell subset tested. Mock-infected targets were used as controls with anti-TCRαβ, anti-Vδ1 and anti-Vδ2 antibodies used to block recognition.

4.6 Discussion

While presumed clinically benign, CMV infection in healthy humans appears to cause major perturbations in lymphocyte subsets over time (Looney *et al.*, 1999, Almanzar *et al.*, 2005, Chidrawar *et al.*, 2009). It would appear that the current study is the first detailed account of how γδ T cell subsets are skewed by the combined effects of aging and CMV carriage in healthy individuals.

The present study observed a dramatic shift towards increased levels of $V\delta2^{neg}$ γδ T cells with an effector memory cell phenotype in CMV-seropositive compared to CMV-seronegative donors. Furthermore, a significant reduction in naive $V\delta2^{neg}$ γδ T cells was found with old age. CMV carriage was associated with reduced naive $V\delta2^{neg}$ cells numbers in each age group, reaching statistical significance in the elderly. These changes in $V\delta2^{neg}$ γδ T cells mirror those described for CD8+ T cells in elderly CMV carriers and thus advance understanding of the host-virus balance that is achieved during normal infection. The current studies also demonstrated $V\delta2^{neg}$ γδ T cells to be overwhelmingly $V\delta1^{pos}$, in agreement with others (Pitard *et al.*, 2008). Increased $V\delta1^{pos}$ γδ T cells have also been reported in HHV-8 infected persons (Barcy *et al.*, 2008). Although HHV-8 status was not tested for, the very low UK prevalence of this virus would suggest it is unlikely to be associated with the observed difference in γδ T cell subsets.

$V\delta2^{neg}$ γδ T cells exceeded 10% of total T cells in several middle-aged and elderly CMV-seropositive donors. Given the shared reactivity of $V\delta2^{neg}$ γδ T cells for tumour cells (Halary *et al.*, 2005), immune responses against malignant cells *in vivo* may contribute towards these T cell expansions. However, the absence of such expansions in

CMV-seronegative donors suggests anti-tumor activity has a limited role. The above findings led us to test for intra-individual associations between $V\delta2^{neg}$ γδ T cell expansion and CMV-specific CD4+ and CD8+ T cells, which are also elevated in the elderly. No correlation was observed between frequencies of $V\delta2^{neg}$ γδ T cells and virus-specific CD4+ T cells and CD8+ T cells within individuals. It is acknowledged that the methods used underestimate the total CMV-specific T cell response; CD4+ and CD8+ T cells are specific for a large number of (>100) CMV antigens (Sylwester *et al.*, 2005), but this would be impractical to measure in a large cohort study such as used here. These CD4+/CD8+ responses are driven by both direct recognition of CMV-infected cells and indirect recognition by cross-presentation of antigens captured by professional antigen-presenting cells (APCs). In addition, CD8+ T cell responses can also be directed against antigens expressed after reactivation from latency, and during latency itself (Blazquez *et al.*, 2010). While $V\delta2^{neg}$ γδ T cells can be triggered by directly infected cells, it is unknown whether viral reactivation from latency or cross-presentation by APCs can lead to efficient stimulation. It will also be important to learn if CMV targets γδ T cells for immune subversion, as is the case for other T cells and for NK cells (Powers *et al.*, 2008).

$V\delta2^{neg}$ γδ T cells and CMV-specific CD4+ and CD8+ T cells have similar characteristics, which are not shared by $V\delta2^{pos}$ γδ T cells. In particular, $V\delta2^{neg}$ γδ T cells were akin to CMV-specific CD8+ T cells; both are mainly T_{EM} cells, including $CD45RA^{high}$ T_{EMRA} cells, with a highly differentiated $CD27^{low}CD28^{low}$ phenotype. $CD45RA^{high}$ and $CD45RA^{low}$ memory T cells are reported to indicate quiescent and recently activated T cells respectively (Carrasco *et al.*, 2006). It could be that high CD45RA expression by

Vδ2^{neg} γδ T cells reflects non-recent activation of Vδ2^{neg} γδ T cells as low levels (<15%) of CD38 and HLA-DR activation markers were observed in both cell types (data not shown). Vδ2^{neg} γδ T cells also expressed high levels of perforin and granzyme B, similar to both CMV-specific CD8+ and CD4+ T cells, indicating cytotoxic functionality. In contrast, Vδ2^{pos} γδ T cells were mostly CD45RA^{low} (CD45RO^{high}), CD27^{high}CD28^{high} and heterogeneous for perforin and granzyme B expression.

Highly differentiated Vδ2^{neg} γδ T cells appeared to be very stable in number and phenotype during a three-year study period. Vδ2^{neg} γδ T cells were also detected after primary infection in adults, though not at the very high frequencies described in pharmacologically immunosuppressed transplant patients (Dechanet *et al.*, 1999b). In the acute phase the response was mainly composed of T_{EM} (CD45RA^{low}) and T_{EMRA} (CD45RA^{high}) cells, but this response had rapidly contracted and shifted to an overwhelmingly T_{EMRA} phenotype with a concomitant shift toward end-stage highly differentiated cells. In contrast no significant change in phenotype of Vδ2^{pos} γδ T cells was observed. No doubt this analysis was weakened by limited patient numbers nevertheless the findings are consistent with those during acute infection in immunosuppressed CMV-infected transplant patients and CMV-infected newborns (Pitard *et al.*, 2008, Knight *et al.*, 2010, Vermijlen *et al.*, 2010).

Cytokines, such as IL-7 and IL-15, are known to support memory T cell survival and proliferation (Becker *et al.*, 2002, Kaech *et al.*, 2003, Purton *et al.*, 2007) but their contribution to γδ T cell memory is unclear. While it was not possible to determine IL-15R expression by flow cytometry IL-7Rh expression was detected by a minority of Vδ2^{neg} γδ T cells (at similar levels described for CMV-specific CD8+ T cells), whereas

the majority of Vδ2^{neg} γδ T cells T cells were IL-7Rh^{pos} in CMV-seronegative donors. Most CD8+ T cells specific for persistent viruses are non-responsive to these cytokines unless also stimulated through the TCR, suggesting that antigen, and not cytokines, is the primary driving force behind T cell expansion (van Leeuwen *et al.*, 2005, Shin *et al.*, 2007). Furthermore, T_{EMRA} cells appear to be more long-lived than CD45RO^{pos} T_{EM} cells (Wallace *et al.*, 2004). Thus it might be possible that Vδ2^{neg} γδ T cell expansions are also driven more by TCR:ligand interactions than by cytokines and are maintained by increased lifespan rather than greater levels of proliferation.

The reactivity of Vδ2^{neg} γδ T cells against CMV-infected target cells was confirmed using in vitro expanded γδ T cell lines. Of particular note, this was also demonstrated using T cell lines expanded from CMV-seronegative donors. This indicates that CMV-reactive Vδ2^{neg} γδ T cells are present in CMV-seronegative donors, albeit at much lower precursor frequencies, and can be successfully expanded in vitro. This state of readiness provides support for these cells having a central role in the immune response after primary infection. However, it was not possible to demonstrate immediate effector activity in *ex vivo* assays, which was unexpected given the shared effector memory phenotype of Vδ2^{neg} γδ T cells and virus-specific αβ T cells. Being a distinct T cell lineage αβ T cells may require an additional activation signal but the observed result could also be a reflection of the experimental conditions; CMV-infected fibroblasts, while able to sensitize virus-specific CD4+ and CD8+ T cells, may not have expressed sufficient levels of the ligand for optimal stimulation of freshly isolated Vδ2^{neg} γδ T cells. This is supported by very recent work of Willcox and colleague, who have demonstrated that the TCR of γδ T cells (Vγ4Vδ5 clone) can directly bind endothelial protein C

receptor (EPCR), which is a major histocompatibility complex–like molecule restricted to endothelial cells (Willcox *et al.*, 2012). EPCR is upregulated after cellular transformation, with higher expression in carcinomas (Scheffer *et al.*, 2002) and therefore, it can be considered as signature of cell stress. Nevertheless, this binding only occurs when costimulatory molecules are up regulated by CMV infection (Willcox *et al.*, 2012). This supports the notion that γδ T cells required a multimolecular stress signature composed of TCR ligand and costimulatory ligands. However, the single clone studied expressed an unusual Vδ5 encoded receptor, which may not be representative of the major Vδ1+ population of $V\delta2^{neg}$ γδ T cells.

Another possible explanation of why freshly isolated $V\delta2^{neg}$ γδ T cells can not respond to CMV-infected fibroblasts is that they are driven to exhaustion as described for CMV-specific αβ T cells in the elderly (Ouyang *et al.*, 2004, Pawelec *et al.*, 2005, Hadrup *et al.*, 2006) and CD8+ cells with the $CD28^{low}/CD57^{high}$ phenotype (Brenchley *et al.*, 2003). Nevertheless, it is worth mentioning that there is contradictory evidence about the ability of highly differentiated CMV-specific αβ T cells to regain their ability to proliferate (Waller *et al.*, 2007, Chong *et al.*, 2008). Therefore, further work is necessary to clarify whether $V\delta2^{neg}$ γδ T cells show functional impairment in the elderly.

It has to be noted that these experiments are based solely on circulating γδ T cells in the blood. As cells from mucosal sites, where $V\delta2^{neg}$ γδ T cells are likely to be functionally more active, have not been analyzed, some caution is warranted when assessing function. However, based on the knowledge that $V\delta1^{pos}$ cells are mainly located at epithelial tissues, it can be hypothesized that donors with high levels of $V\delta2^{neg}$ γδ T cells in the blood may have even higher levels of $V\delta2^{neg}$ γδ T cells at these sites possibly driven by

an elevated degree of localized viral replication.

Studies of TCR usage by Vδ2^{neg} γδ T cells populations show greater TCR restriction in CMV-seropositive individuals compared to CMV-seronegative controls (Dechanet *et al.*, 1999a, Pitard *et al.*, 2008, Knight *et al.*, 2010), indicating an antigen-driven selection of T cell clonotypes as observed for CMV-specific CD8+ and CD4+ T cells (Price *et al.*, 2005, Trautmann *et al.*, 2005). Indeed, a recent study describes a public Vδ1 clonotype emerging during CMV infection *in utero* (Vermijlen *et al.*, 2010). While TCR usage was not determined, it is tempting to speculate that public Vδ1 TCRs are also present in some donors. This clonal focusing may be driven by the selection of higher affinity TCRs (Price *et al.*, 2005, Trautmann *et al.*, 2005). Formal identification of the ligand(s) for the TCR of these γδ T cells would help answer some of these questions and also be of immense value for vaccine design, a major priority for CMV (Arvin *et al.*, 2004), where the aim should be to elicit broad multi-specific immunity (Khanna and Diamond, 2006).

Acknowledgment

Appreciation goes out to Mustafa Halawi who intensively participated during his master course in establishing the γδ T cell lines and CMV-recognition assay.

5 Role of Cytomegalovirus in Coronary Artery Disease

5.1 Background

Coronary Artery Diseases (CAD) (also called Ischemic heart diseases IHD) are the leading cause of death in adulthood worldwide. The pathological change behind these diseases is stenosis of coronary arteries due to atherosclerosis and plaque formation. It is multifactorial disease and there are several risk factors for atherosclerosis such as smoking, hypertension, diabetes mellitus, and hyperlipidemia (Ellenbogen, 1996). However, in recent years, there has been increasing focus on the role of chronic inflammation in the pathogenesis of atherosclerosis (Khan *et al.*, 2011). One of the proposed causes of inflammation is chronic bacterial and viral infection. Although CMV was near the top of the causative agents list, there has been conflicting data as to whether CMV is a cause or an effect of atherosclerosis and CAD.

There are several prior studies that have noted the importance of CMV in the etiology of CAD. For instances, in 1996 Zhou *et al* demonstrated that CMV carriage increased the risk of coronary artery restenosis by clot or plaque reformation after it had been removed during cardiac catheterization (Zhou *et al.*, 1996). Subsequently, in 1998 Blum *et al* showed that high levels of anti-CMV IgG titre correlate positively with prevalence of coronary artery disease (Blum *et al.*, 1998). Furthermore, Neumann and colleagues in 2000 showed that there is a high risk of complication such as re-infarction, urgent re-intervention and even higher rate of death in CMV seropositive patients in the thirty days post stent placement compared to CMV seronegative patients (Neumann *et al.*, 2000). In 2007 Gredmark *et al* utilized RT-PCR to show that there is a high rate of CMV replication in peripheral blood of patients with coronary disease compared with healthy controls (Gredmark *et al.*, 2007). Nevertheless, in the same year Iriz's group could not

detect CMV infection by PCR when they examined aortic and internal mammary artery biopsy specimens from 33 patients who underwent open-heart bypass surgery (Iriz *et al.*, 2007). Contrary to expectations, Xanaki's group were able to detect CMV DNA in endothelial cells of coronary arteries. However there was no significant difference in viral load in nonatherosclerotic tissues and atherosclerotic plaques in the group of patients who had bypass surgery (Reszka *et al.*, 2008).

No doubt such contradictory findings about CMV as a causative agent in CAD necessitates a sensible explanation for the CMV and CAD association. One possible explanation is based on the concept of immune system modulation such immunosenescence by chronic infection with CMV (see section 1.2.5 and 1.2.8 in general introduction). For instance, there is a considerable amount of evidence for continuous immune activation in CAD which is characterized by accumulation of immunocompetent cells in the arterial lesion and elevation of inflammatory markers in the circulation (Hansson *et al.*, 1989, Caligiuri *et al.*, 1998, Mazzone *et al.*, 1999, Jonasson *et al.*, 2002, Khan *et al.*, 2011) and CMV is believed as the trigger antigen for this persistent immune activation. This is consistent with the fact that CMV is never cleared from the host, and enters into latency with poorly understood periodic reactivation and viral replication, which is controlled by reactivation of memory T cells (Sinclair and Sissons, 2006, Reeves and Sinclair, 2008, Sinclair, 2008). This notion is supported by the coexistence of inflammation and seropositivity for CMV as the most important predictor for reduced survival in CAD. In other words, patients who were CMV seropositive without coexisting inflammation did not show adverse prognosis (Zhu *et al.*, 1999, Muhlestein *et al.*, 2000, Simanek *et al.*, 2011).

Besides the chronic CMV seropositivity and state of persistent inflammation, a third factor, leukocytes telomere length, has become an important component in understanding the relationship between infection and CAD. For instance, individuals with shorter telomeres in their peripheral blood leucocytes carry a higher risk of dying from cardiovascular disease (Samani *et al.*, 2001, Nowak *et al.*, 2002, Cawthon *et al.*, 2003, Yamada, 2003, Brouilette *et al.*, 2007). On the other hand, CMV seropositive patients, but not healthy controls, exhibit accelerated telomere shortening of their cytotoxic T lymphocytes (CTLs) (Spyridopoulos *et al.*, 2009, van de Berg *et al.*, 2010, Spyridopoulos, 2011). Over time, the persistent antigen exposure would induce not only temporary change in T cell activation markers but also with each cell division cycle, the telomere becomes shorter until the cell reaches the terminal effector stage (Blasco, 2005) and permanent alteration in the T cell phenotype pattern could occur (Effros *et al.*, 1996, Monteiro *et al.*, 1996, Valenzuela and Effros, 2002, Fletcher *et al.*, 2005). In immunologica1 terms this stage is known as the immunosenescence stage (Kaszubowska, 2008). This stage is characterized by poor response of the immune system to antigens and is associated with morbidity and mortality (Alonso-Fernandez and De la Fuente, 2011, Stankiewicz and Stasiak-Barmuta, 2011). Relation between CMV carriage and immunosenescence is reviewed in section 1.2.7 and 1.2.8 in the introduction chapter.

It is worth mentioning that the quality of the immune response mounted against an antigen depends not only on the frequency of T cells reactive against that antigen but also on their functional pattern (Betts *et al.*, 2006, Darrah *et al.*, 2007, Seder *et al.*, 2008). In this regard, far too little attention has been paid to the relationship between

the change in the magnitude of CMV specific T cell response and prognosis in coronary artery disease patients.

Hypothesis

Here it is hypothesized that persistent subclinical CMV reactivation in ischemic heart disease patients leads to a stage of immunosenescence that particularly affects CMV specific T cells. Based on the observation that CMV specific T cells comprise a relatively large percentage of the memory T cell pool, the functional quality of CMV specific T cells must be an important factor in the morbidity and mortality in coronary heart diseases.

5.2 Aims

It is not known whether the frequency and functional integrity of CMV reactive T cells in patients with coronary artery disease (CAD) differ from those of otherwise healthy subjects, and whether CMV specific T cells in CAD show premature signs of exhaustion that are represented as oligo-functionality, which in this thesis is referred to concurrent expression of two or less of the measured markers by CMV-specific T cells. Hence the aim is to determine differences in the magnitude of T cell responses to CMV in CAD and age-matched healthy volunteers by 8-colour flow cytometer.

5.3 Plan of investigation

To achieve the current project aims a plan of analysis was designed to include five steps, see Figure 5.1. The initial step was to carry out optimization experiments, this followed by determination of the study population CMV status. Finally, in two separate steps both the frequency and the functional integrity of CMV specific T cells and non-CMV specific T cells were measured *ex-vivo* in all patients and results were compared with age-matched seropositive healthy adults.

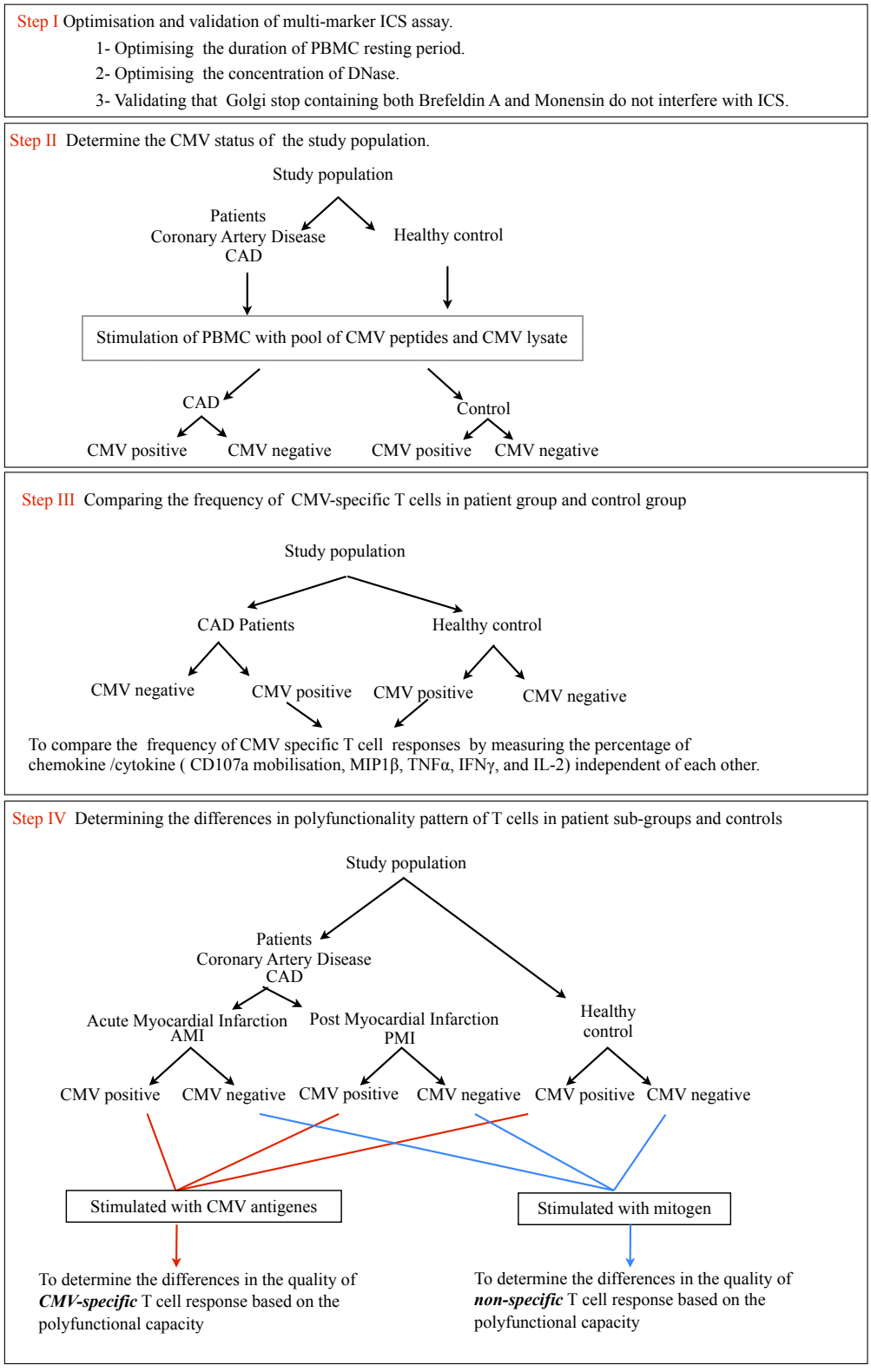


Figure 5.1: Schema for investigation steps

5.4 Results

5.4.1 Study population

Samples were recruited from the Cardiology Department, Newcastle Freeman Hospital. The patient cohort consisted of 57 patients (age 45-65 years) diagnosed with coronary artery disease (CAD) (also known as ischaemic heart disease IHD). 26 of them suffered acute myocardial infarction (AMI) while the rest were in stable post myocardial infarction stage (PMI). The patients selected lacked any history of cancer, inflammatory disease, immune disorders or severe comorbidity. However, all of them were on Aspirin (75-150 mg), Beta-blocker and statins. As a control group 40 age-matched healthy volunteers were used. Ethical approval was obtained from the ethics committee of Freeman Hospital and University of Liverpool.

5.4.2 CMV status of study population

To address the aims it was necessary to confirm the CMV status of the cohort of blood donors. This involved the use of two complementary methods; serological method utilizing ELISA and αβ T cell responses against CMV measured by flow cytometry.

The serological part was carried out by collaborators in the Freeman Hospital, Newcastle. They utilized an ELISA technique to detect CMV specific IgG in serum (data not shown). Out of 57 patients tested there were 28 CMV-seropositive donors according to this assay. As this was not a quantitative assay, information on the level of CMV-specific antibodies could not be generated.

T cell responses against CMV antigens were tested by using CMV lysate plus an inclusive CMV peptide pools, as this has been shown to be a good way to confirm CMV carriage (Kern *et al.*, 2000). These pools spanned the entire amino acid sequences of the

HCMV pp65, major immediate early (IE-1) proteins and DYS/VTE. They comprised of 15-amino acid peptides with at least nine overlaps between neighboring peptides.

T cell responses were measured by staining PBMC with antibodies against T cell markers (CD4 and CD8) and then against intracellular cytokines IFNγ, TNFα, IL-2. Figure 5.2 shows representative examples of donors tested for T cell responses against CMV antigens. The increase in the proportion of any of the mentioned cytokine production by CD4+ T cell or CD8+ T cell was used to decide whether there was response or not, an example of the formula that used to calculate this proportion is shown below. Since not all CMV seropositive donors (based on ELISA) showed T cell responses the conclusion on CMV status was made using both assays; see Table 5-1 below.

Frequency of CMV-specific CD4+ or CD8+ T cells based on IFNγ production=

% $IFN\gamma^{pos}$ (CD4+ or CD8+) T cells events in response to mock.

Subtracted from

% $IFN\gamma^{pos}$ (CD4+ or CD8+) T cells events in response to CMV-antigen

An example applying the above formula on pt007 is shown below.

% CMV specific CD4 T cells out of total CD4+ T cell in CMV stimulated tube ⇨ (0.751/45.05)X 100 ≈1.66

(i.e IFNγ+ CD4+ T cells) (−)

% CMV specific CD4 T cells out of total CD4+ T cell in mock stimulated tube ⇨ (0.0406/61.34)X100 ≈ 0.066

Frequency of CMV-specific CD4+ T cell≈ 1.59

Table 5-1 : Examples of CMV status of donors tested in this study

Donor ID	Clinical condition*	CMV-specific antibodies (ELISA)	IFN-γ response after CMV stimulation** (Flow cytometry)	CMV status
P001	AMI	?	0.9%	Positive
P003	AMI	+	1.58%	Positive
P007	AMI	+	1.53%	Positive
P008	AMI	+	0.6%	Positive
P009	AMI	_	0.1%	Negative
P010	PMI	+	0.1%	Positive
P028	AMI	-	0.1%	Negative
P016	PMI	+	1.67%	Positive
P044	AMI	_	0.0%	Negative
P051	PMI	+	2.1%	Positive
P058	PMI	-	2.3%	Positive

* AMI= Acute Myocardial Infarction, PMI= Post Myocardial Infarction

**IFNγ response is calculated after subtracting the response against mock lysate.

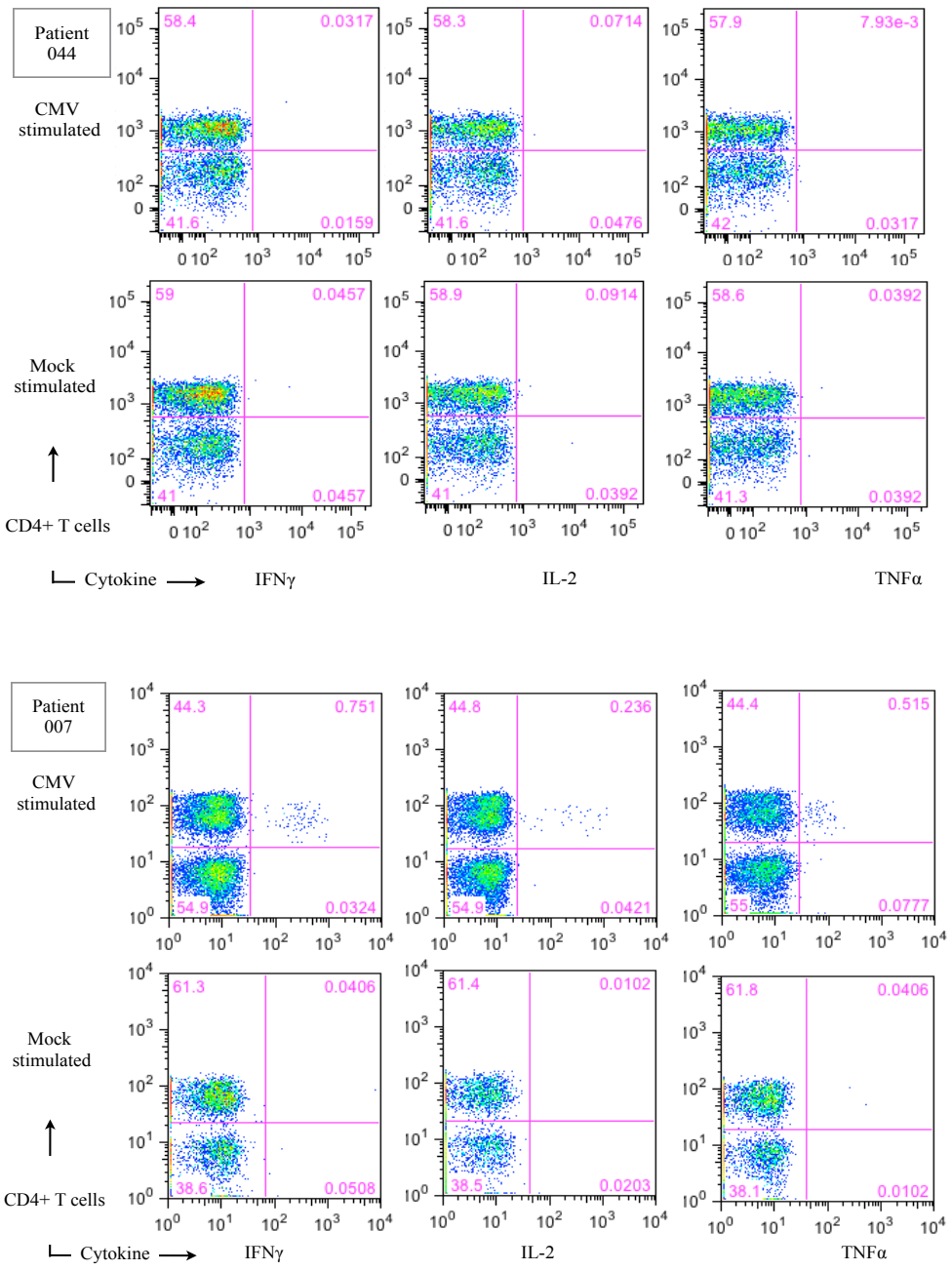


Figure 5.2: Representative flow cytometry plots of negative and positive blood donor responses against CMV antigens.

This figure shows two representative results of patient's responses against CMV. The upper groups of plots are for a CMV seronegative patient (Pt 044) while the lower are from a CMV seropositive patient (Pt 007). PBMC were stimulated for 10 hours at 37°C followed by surface staining for CD4 and then intracellular staining for IFNγ, , TNFα , and IL-2. The frequencies shown in the quadrants refer to the percentage of total lymphocytes.

5.4.3 Optimization of the ICS assay

Since eight-colour flow cytometry for intracellular cytokine staining (ICS) on frozen peripheral blood monocyte (PBMC) is potentially demanding, a preliminary set of standardization steps was carried out before doing actual experiments on the patients' samples. Therefore, in addition to the ordinary optimization steps such as, choosing which fluorochromes and use of isotype monoclonal antibody controls to each marker and titration of antibody quantity to reduce the background noise, the following were done:

1- Incorporation of eight to ten hours PBMC rest period.
2- Addition of DNase to the growth media during washing steps and subsequent incubations.
3- Selection of appropriate cytokine secretion inhibitors to be used in ICS.

5.4.3.1 PBMC rest period and the addition of DNase to the culture medium

It is known that freezing and thawing is very stressful to PBMC. Therefore incorporating a resting period for PBMC before commencing stimulation and ICS steps is favourable (Gauduin *et al.*, 2004, Nomura *et al.*, 2008). In addition, using DNase during washing steps was found to remarkably decrease PBMC stickiness (Lamoreaux *et al.*, 2006, Horton *et al.*, 2007). Thus, both of these approaches were adopted in the current study. However, the length of the PBMC resting period is not precisely determined and it varies according to the purpose of the experiment; besides, the possible effects of DNase on the ICS assay are unknown. It was therefore essential to optimize the length of PBMC resting period and to determine the appropriate concentration of DNase needed to be used in the ICS assay. Herein, flow cytometry was used to determine the level of IFNγ production by CD4+ and CD8+ T cells upon stimulation with CMV antigens in the

presence of different concentrations of DNase and for different incubation periods. The initial step was to find the optimal concentration of DNase to be used; to do that four vials of frozen PBMC from healthy donors known to be CMV-seropositive were thawed. One vial was washed with and cultured in RPMI medium alone (concentration 1-2×10^6 PBMC/1 ml medium). Meanwhile, the other vial was aliquoted into three test tubes each contains 1-2×10^6 PBMC in 1 ml medium; three different concentrations; 1:10, 1:100 and 1:1000 of DNase in RPMI medium were used for washing and incubation of these aliquots. After resting at 37 ^{O}C in a 5% CO_2 incubator for fifteen hours, cells were washed twice with FACS buffer then recounted and any cell aggregations or clumps were removed. This was followed by staining with FITC conjugated anti-CD4 and PE conjugated anti-CD8 monoclonal antibodies. Finally, fixation, permeabilization and intracellular staining of IFNγ with anti IFNγ APC conjugated monoclonal antibody were preformed (For more details see section 2.4.3.).

The preliminary observation was that the DNase treated samples showed higher yields of PBMC. In addition, there was notable reduction in the background staining in mock-stimulated tubes treated with DNase. Nonetheless, there was no significant change either in T cell subsets frequency or in the level of IFNγ in CMV stimulated tubes (Figure 5.3). The next step was to determine the optimum duration for resting of PBMC without stimulation and in the presence of DNase. Therefore, resting of cells for six, ten and fourteen hours were carried out before stimulation and ICS steps. There were no remarkable differences in levels of cytokine production (data not shown). However, for convenient scheduling ten to fourteen hours (i.e. overnight) were chosen as an optimal incubation period.

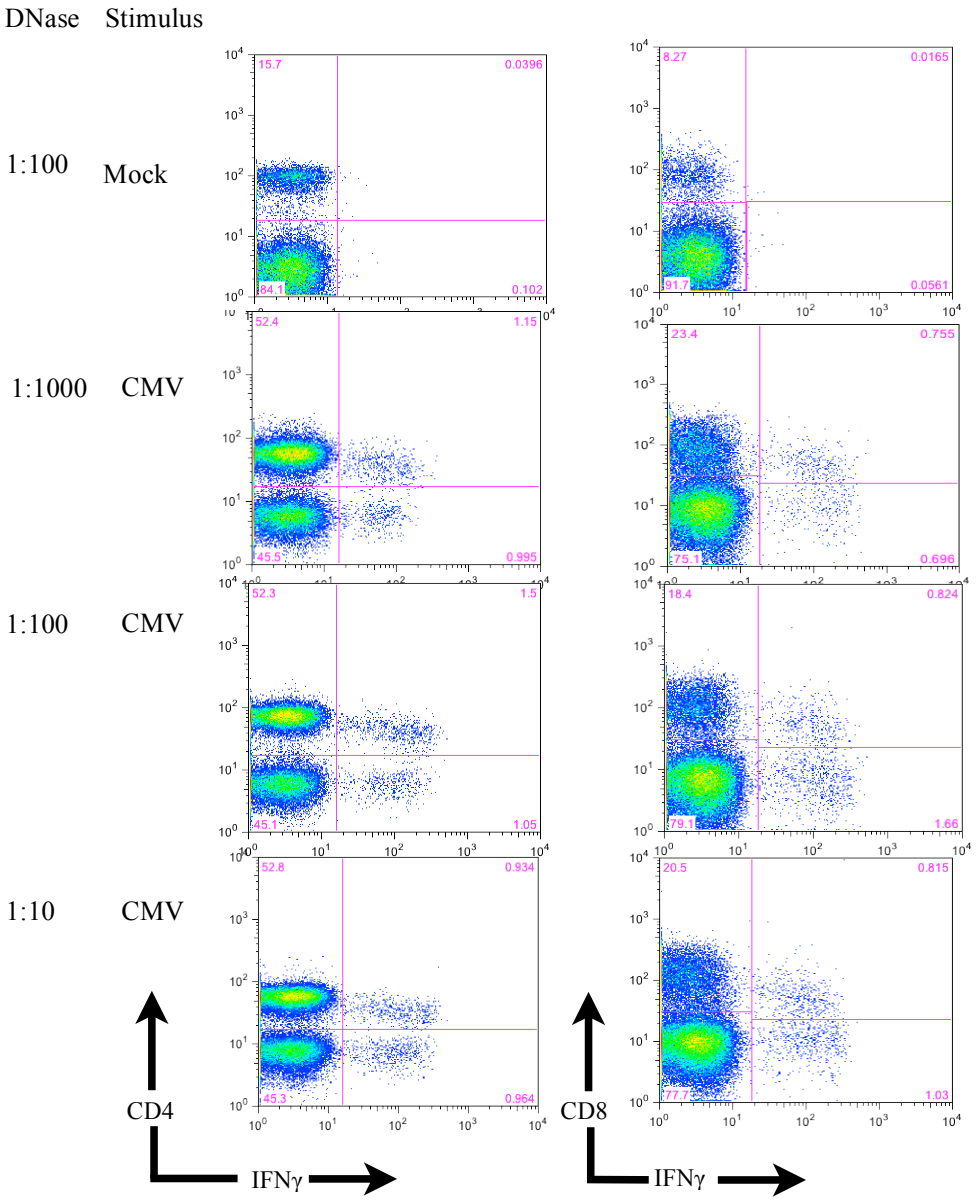


Figure 5.3: DNase does not interfere with the cytokine detection assay.

It demonstrates the response of CD4+ and CD8+ T cells (left and right panels of plots, respectively) to mock and CMV antigens in the presence of different DNase concentrations. The top row of plots show the response of T cells to mock, whereas the low three rows of plots reveal T cells responses to CMV antigens. The Y-axis represents T cells frequency while the X-axis demonstrates IFNγ production in both CD4+ and CD8+ T cells. Labels and numbers to the left of the Y-axis show the stimulus type and DNase concentration used in each set of experiments.

5.4.3.2 Golgi Stop containing monensin enhances the CD107 mobilization assay detection threshold.

Appropriate measuring of T cells response should include assessment of the ability of cells to kill (cytotoxic capability). This can be achieved either by directly measuring intracellular cytotoxic granule contents such as perforin and granzymes or indirectly by measuring degranulation marker such as CD107a or CD107b. Implementing the last option necessitated the use of Golgi Stop containing monensin besides brefeldin-A (BFA). Aiming to find out the effects of cytokine secretion inhibitor on the ICS assay, PBMC from a CMV-seropositive donor were aliquoted into four test tubes. One of the tubes was stimulated with mock infection while the others were stimulated with CMV antigens. Immediately after stimulation, PBMC were stained with FITC conjugated anti-CD107a/b monoclonal antibody in the presence or absence of monensin, followed by 10 hours incubation in growth media containing co-stimulatory antibodies (pure anti CD28 and anti CD49d) and BFA. On day two the surface and intracellular staining were carried out and flow cytometric analysis done. Figure 5.4 is an illustrative example of how monensin could dramatically enhance the detection threshold of the CD107 mobilization assay.

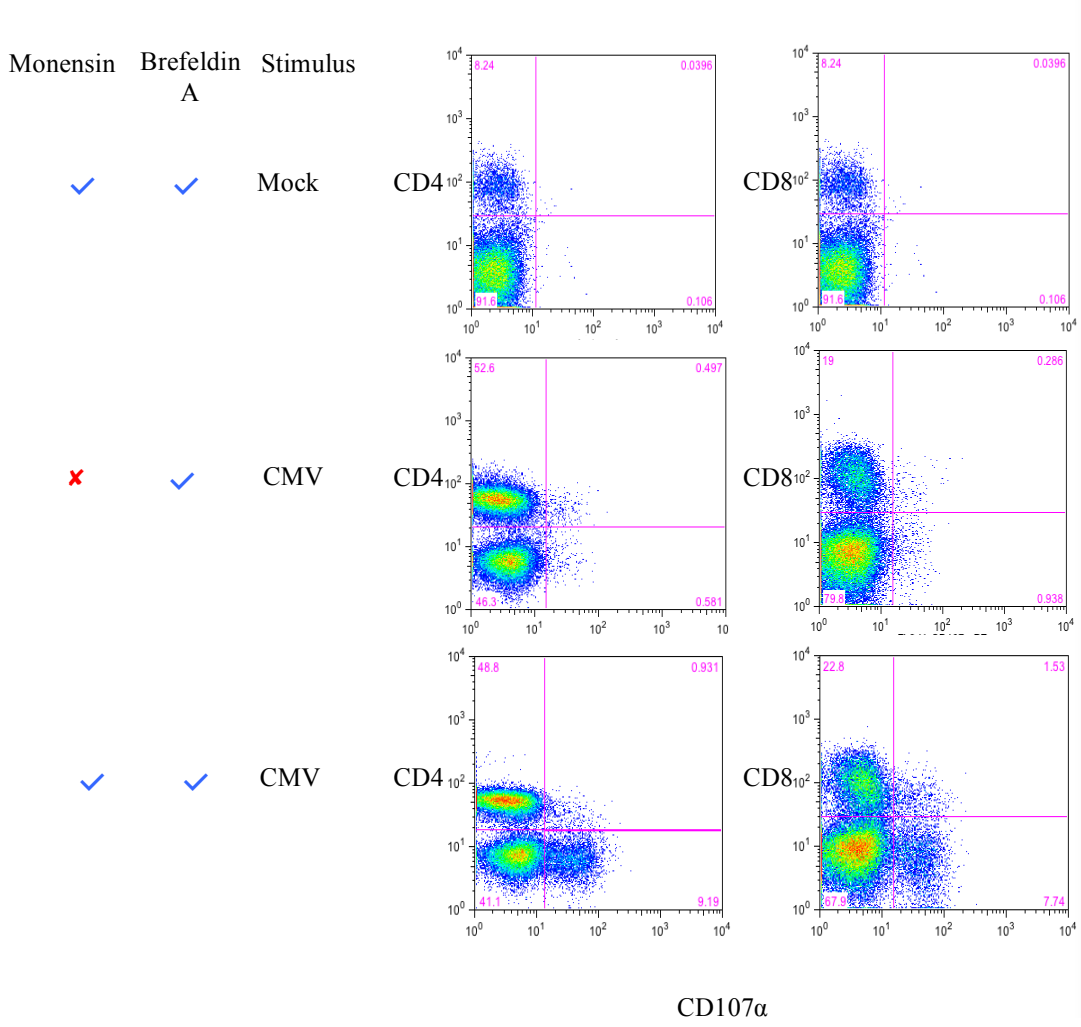


Figure 5.4: Golgi stop containing monensin increases the sensitivity of CD107a detection.

Illustrative example of the influence of Golgi stop containing monensin on the level of CD107a expression of CMV stimulated T cells. PBMC incubated with mock (top row of plots) and CMV (second and third rows of plots) labelled with anti-CD107a monoclonal antibody. Monensin was used with the mock infection and in the lower plots only. Here, the Y-axis of each plot denotes either CD4+ or CD8+ T cell frequencies while the X-axis shows the level of CD107a expression.

5.4.4 Concurrent measurement of five separate T cell functions

To determine the polyfunctionality of CMV specific T cell responses, simultaneous and independent measurements of CD107a mobilization and MIP1β, TNFα, IFNγ, and IL-2 production were carried out. The gating scheme for identification of polyfunctional T cells is shown in Figure 5.5. Briefly, PBMC from a CMV seropositive CAD patient were stimulated with a mixture of IE-1 and pp65 CMV peptides and CMV lysate for approximately 10 hours in the presence of co-stimulatory antibodies, Golgi inhibitor and anti CD107a monoclonal antibody. This was followed by intracellular staining with anti MIP1β, IFNγ, TNFα, and IL-2 monoclonal antibodies to determine the functional capacity of T cells. In this subject, both the CMV specific CD4+ and CD8+ T cells demonstrated each of the five functions. For instance, the percentage of CD4+ T cells that produced CD107a was 2.31%, while for other cytokine production it ranged from 0.6% to 2.13%. In contrast, the frequency of CD107a+ CD8+ T cells were 1.45 of the gated PBMC (plots not shown); with other functions having slightly lower frequency ranges than for CD4+ T cells.

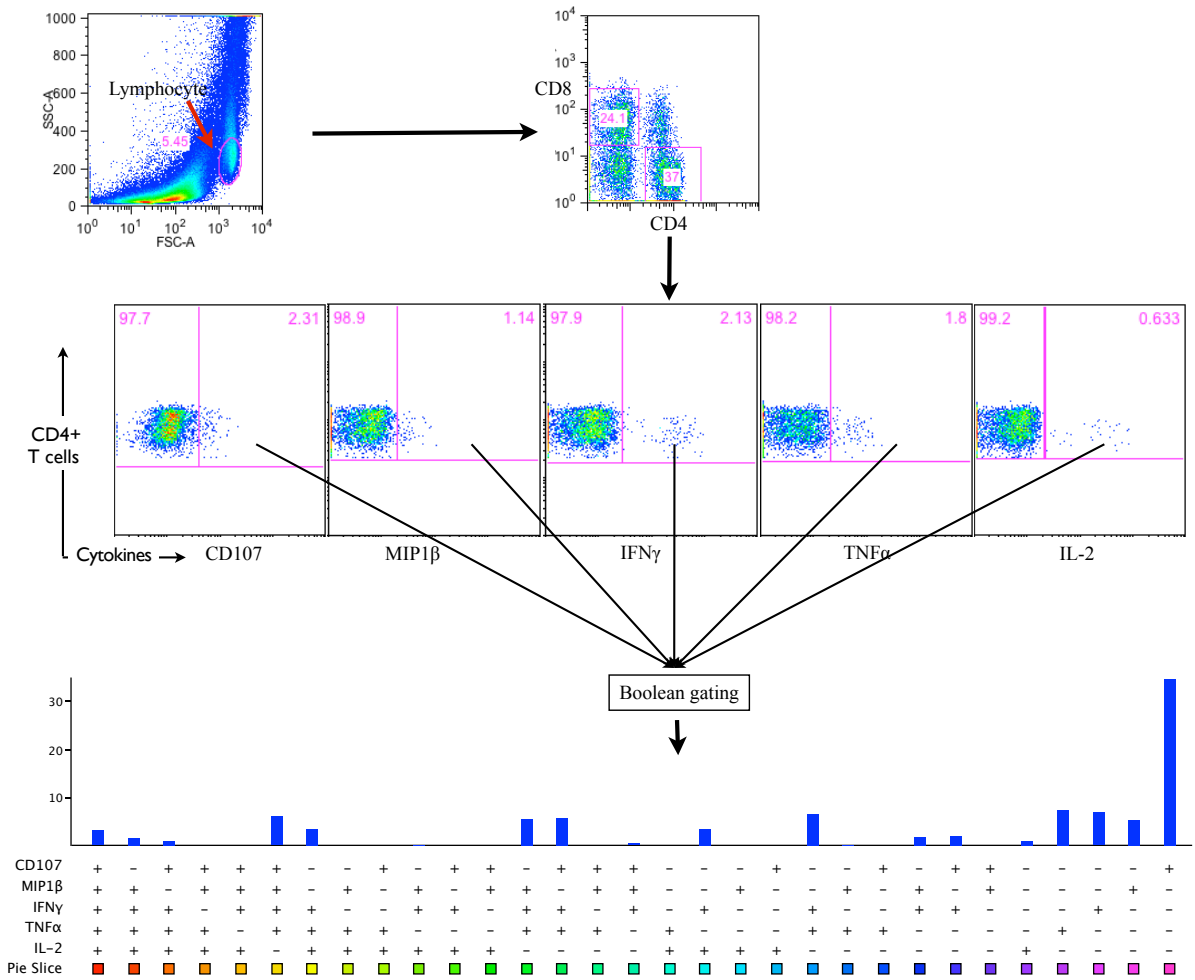


Figure 5.5: Detection of five T-cell functions and characterization of CMV-specific T cell polyfunctionality in coronary artery disease.

This portrays gating steps for identification of multifunctional CD4+ T-cell responses. The top two plots illustrate the progressive gating strategy used to identify the lymphocyte population. Meanwhile, the bottom panels represent results of the five functional responses of the CD4+ T cells to CMV antigens. The histogram summarizes the 32 possible polyfunctional combinations of CMV specific CD4+ T cells. Data demonstrated are from a CMV-seropositive patient with coronary artery disease.

5.4.5 CMV specific CD8+ T cells in patient groups exhibit higher levels of inflammatory cytokines compared to the control group.

Determination of CD8+ T cell responses to CMV by measuring the frequency of a single marker independent of other functional markers showed that the patient group usually demonstrated a higher frequency of responding CD8+ T cells compared to controls (Figure 5.6). This difference was obvious among IFNγ and TNFα positive CD8+ T cells where P values were <0.05 and < 0.01 respectively. Since the patient group consists of two categories of patients, namely patients with Acute Myocardial Infarction (AMI) and patients with stable Post Myocardial Infarction (PMI), then it would be more rigorous to compare these subgroups of patients to each other rather than only to healthy donors (Figure 5.7). Statistically significant differences in frequency of TNFα+ CD8+ T cells can be seen between PMI and control groups ($P<0.01$) and in CD8+ IFNγ+ double positive T cells between AMI and control groups ($P<0.05$).

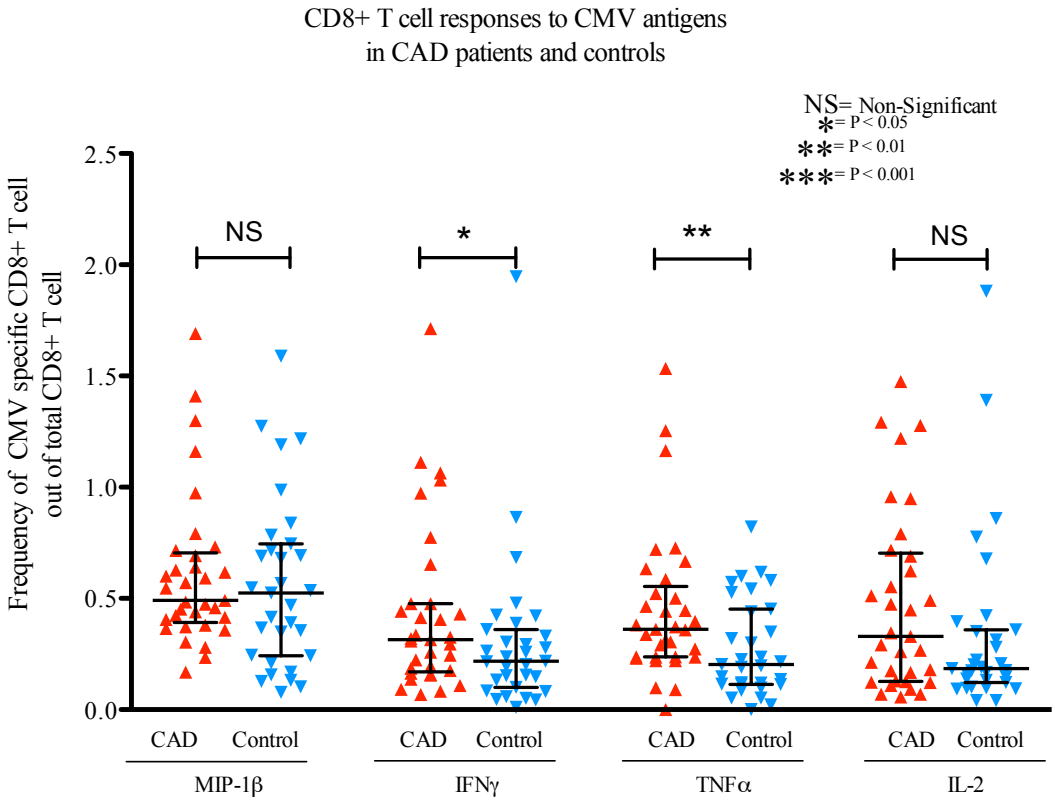


Figure 5.6: CD8 T-cell responses to CMV antigen in control and patient groups.

Scatter dot plot comparing the frequency of CMV specific CD8+ T cells out of total CD8+ T cells between patients with coronary artery disease (CAD) and control groups, note both groups here are CMV seropositive. Blue triangles denote control groups, while red triangles represent patient groups. The bars indicate the medians with interquartile ranges. The X-axis indicates which function was measured (MIP1β, IFNγ, TNFα, and IL-2) while the Y-axis indicates the percentage of specific CD8+ T cells out of total CD8+ T cells. Note, asterisks at the top of the plot indicate the significance of the difference between the groups, see figure label.

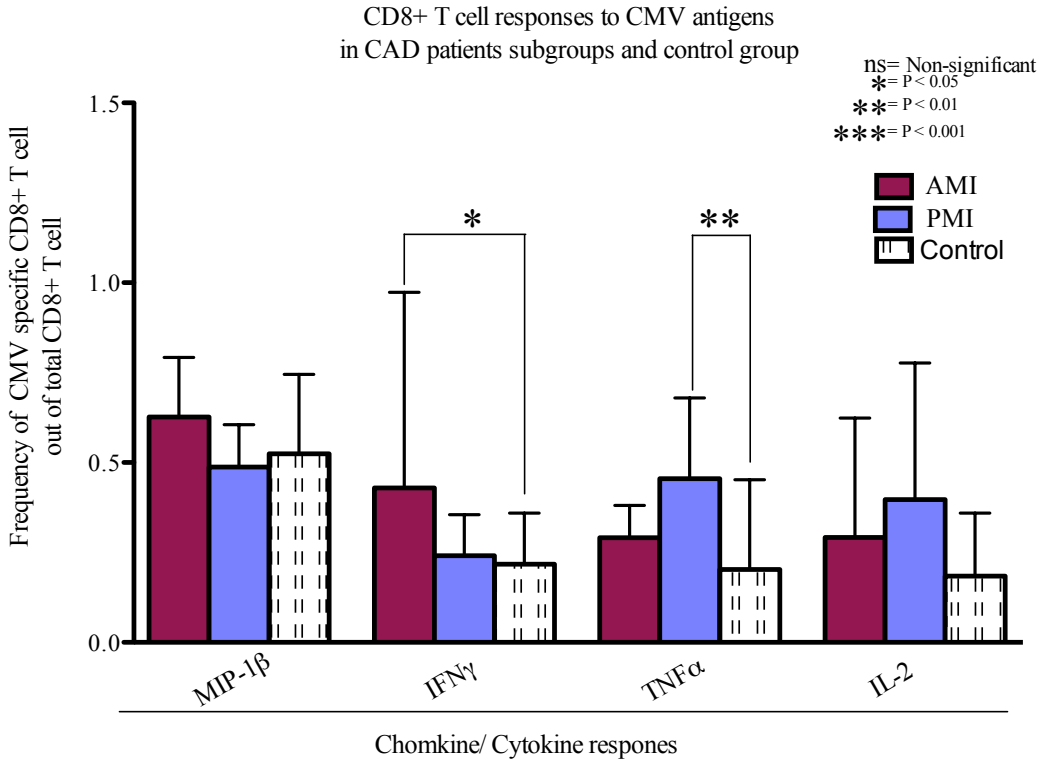


Figure 5.7: Comparison of the frequencies of CD8+ T cell responses to CMV antigens among different patient subgroups.

Comparison between the frequencies of CMV specific CD8+ T cells out of total CD8+ T cells (Y-axis) in coronary artery disease (CAD) patient subgroups and the control group. From right to left, the color-coded bars are referring to: patients with acute myocardial infarction (AMI), patients with stable post myocardial infarction (PMI) and control group respectively. The height of bars and error bars indicate the median and upper-quartile range respectively. The four functions measured, MIP1β, IFNγ, TNFα, and IL-2 are shown on the X-axis, and the asterisks at the top of bars refer to the significance of the difference between the groups (see figure legend).

5.4.6 Similarity in the frequency of CMV specific CD4+ T cells in patient and control groups.

Although the response patterns of CD4+ T cells to CMV antigens in patient and control groups were similar to that of CD8+ T cells, there were no significant differences in frequencies of CMV-specific CD4+ T cells between patient and control groups. For instance, the independent measurement of five functional markers in CD4+ T cells after stimulation with CMV antigen revealed no substantial variation between the patient and control groups (Figure 5.8). Furthermore, no statistical difference emerged even when the patient groups were clinically categorized into their subgroups (AMI and PMI) and comparison preformed at the level of these subgroups (Figure 5.9).

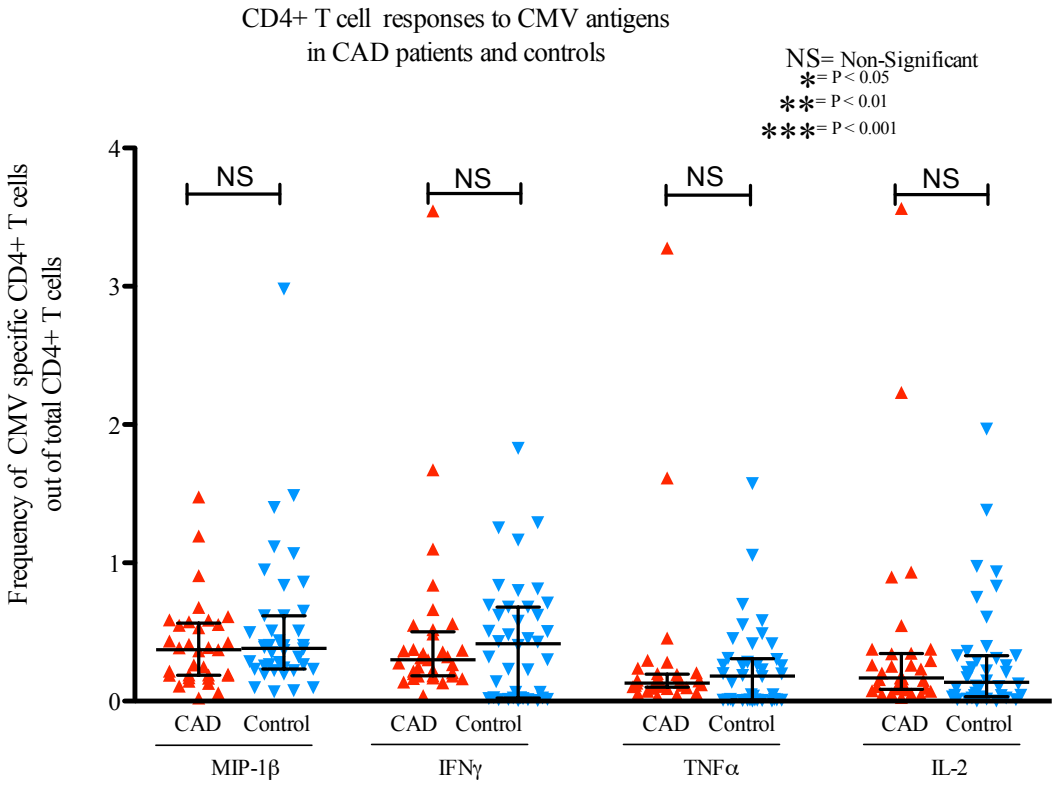


Figure 5.8: Resemblances in frequencies of CMV specific CD4+ T cells of patient and controls groups.

Figure demonstrates the similarity between patient and control group (red and blue triangles respectively) considering the percentage of CD4+ T cells that respond to the CMV antigens (Y-axis) by expressing one of the functional markers; MIP1β, IFNγ, TNFα, and IL-2 (X-axis). The bars are indicating the median with interquartile range.

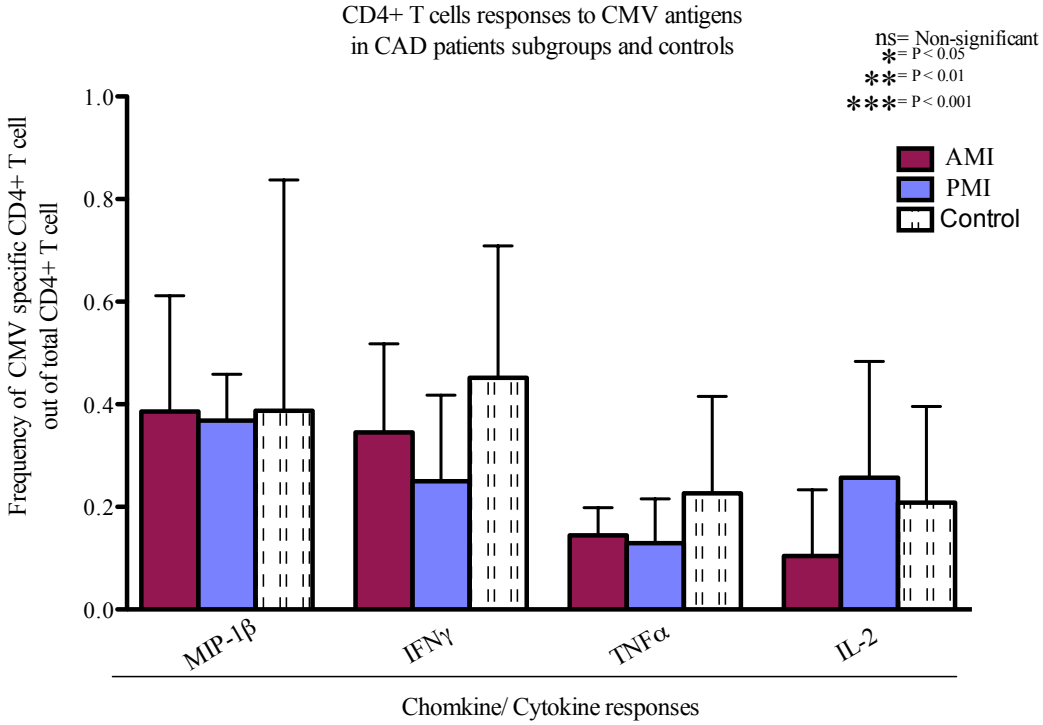


Figure 5.9: Comparing the frequency of CD4+ T-cell responses to CMV antigen in patient subgroups (AMI &PMI) and the control group.

Comparison of the frequency of CMV specific CD4+ T cells out of total CD4+ T cells (Y-axis) among AMI, PMI and control groups (red, blue, and clear-pattern bars respectively). The height of bars and error bars indicate the median and upper-quartile range respectively. Despite the apparent differences within some clusters, particularly for IFNγ and IL-2, no statistical differences were found.

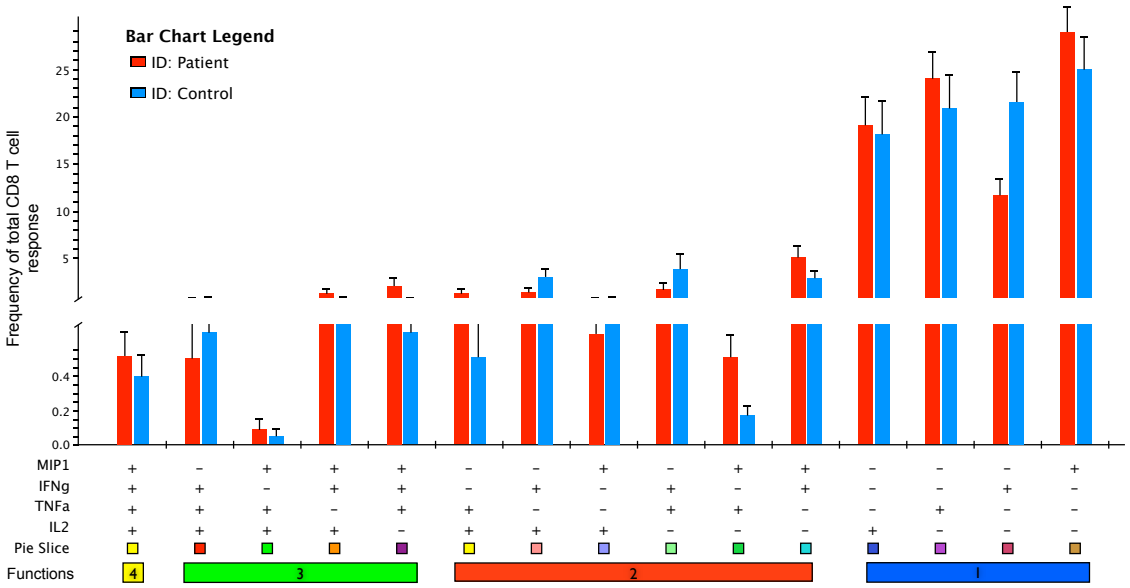
5.4.7 Measurement of T cell functional capacity based on Boolean gating of different functional markers

Aiming to measure the full complexity of T cell response Flowjo software (version 9.4.10;Mac) was used to produce an array of Boolean gates of five response markers, which include CD107a, MIP1β, IFNγ, TNFα, and IL-2. This resulted in the generation of 32 possible combinations of these five functions (Figure 5.5). With such a large database it was essential to use database organizing software. Thus, PESTLE software was utilized to reorganise the data and correct any non-specific background responses by subtracting the mock responses (negative control) from both the CMV antigen and mitogen (CON A as a positive control) responses. Finally, data were exported to a special program called ***SPICE*** 5 (*Simplified Presentation Incredibly Complex Evaluations*) (Roederer, 2011) where graphs and some statistical work were produced. Notably, most of the intracellular stains were satisfactory; nevertheless, CD107-FITC was substandard with high background levels. Therefore, CD107 was purposely excluded from the Boolean gates to prevent any statistical biases. Consequently, CD107 will neither appear in graphs nor is included in the statistical analysis. Accordingly the number of possible combination automatically was reduced to 15 instead of 32 combinations.

5.4.8 CD8+ T cell response to CMV is mostly limited to cell populations with restricted functionality

Investigating the functional hierarchy of CMV specific CD8+ T cell response in both patient and control groups (Figure 5.10) showed that most responses were restricted to a

single functional marker. For instance, in the patient group, CD8+ T cell responses were mainly dominated by cells that only produce MIP1β followed by TNFα and with less IL-2 preponderance. Meanwhile in the control group IFNγ and TNFα monofunctional cells were positioned second in the order just after MIP1β; again IL-2 secreting cells were the least frequent. In addition, some of the responding populations exhibited multifunction patterns that mostly segregated to 3 or less simultaneous functions. The percentage of contribution of each of these combinations in total CD8+ responding cells was comparable and ranging from 0.4% to 4% of total CD8+ T cells response with the exception of the MIP1β+ IFNγ- TNFα+ IL-2+ CD8 T cell combination which was approaching zero. Interestingly, the CD8+ T cell response pattern in control groups was very similar to that of patient groups and there were no statistical significant differences between the two groups.



Functions				P Value
MIP1β	IFNγ	TNFα	IL-2	
+	+	+	+	0.0707
+	+	+	-	0.5804
+	-	+	+	0.3283
+	+	-	+	0.1173
-	+	+	+	0.6753
-	-	+	+	0.2263
-	+	-	+	0.3629
+	-	-	+	0.4798
-	+	+	-	0.3707
+	-	+	-	0.8811
+	+	-	-	0.1580
+	-	-	-	0.7676
-	+	-	-	0.1041
-	-	+	-	0.5809
-	-	-	+	0.4277

Figure 5.10: Comparison between CD8+ T cell functional capacity in patients with coronary artery disease (CAD) and healthy controls.

The graph demonstrates the percentage contribution of each of the fifteen possible combinations of CMV specific CD8+ T cell in response to CMV antigen (Y-axis). Red and blue vertical bars represent the median of these combinations in coronary artery disease patient and healthy CMV carriers, respectively. Each combination of the four functions that involves; MIP1β, IFNγ, TNFα, and IL-2 is colour coded as a small square below the X-axis. For simplicity, the fifteen combinations were regrouped according to how many chemokine/cytokines were produced simultaneously into four major groups. Horizontal bars of different colours below the x-axis were used to portray these major groups. The integrated table below the graph provides the P value differences between patient and control groups of each combination based on the Mann-Whitney test.

5.4.9 Permutation analysis of the patient and control groups demonstrates similar patterns of CD8+ T polyfunctional response to CMV antigens.

The fifteen possible combinations of functions were represented in pie charts (Figure 5.11a) where each slice in the pie charts symbolizes one of the fifteen combinations. Therefore, the colour of each slice matches the colour of the square below the X-axis in the histogram in Figure 5.10. Statistically, the comparison of distributions from individual categories in these complex datasets is subjected to Type 1 statistical error. Therefore, it would be reasonable to reduce the number of comparisons by replacing the fifteen functional combinations with four major function groups. The first group includes CD8+ T cells that express only one function (1+). The second group comprises responding cells with a combination of any two functions (2+); meanwhile the third and fourth groups represent, respectively, any three or four function combinations.

Thereafter, the pie chart was reproduced using only the four major groups (Figure 5.11b). Each section in the new pie chart was colour-coded thus it matches corresponding horizontal bars in the histogram in Figure 5.10. Furthermore, for clarification purposes, arcs were added to each pie chart, where each arc symbolizes a single function. The overlap between arcs determines the pattern of polyfunctionality. By performing a permutation comparison between every pair of pies we found that there was no significant difference ($p=0.1375$) between the patient and control cohorts.

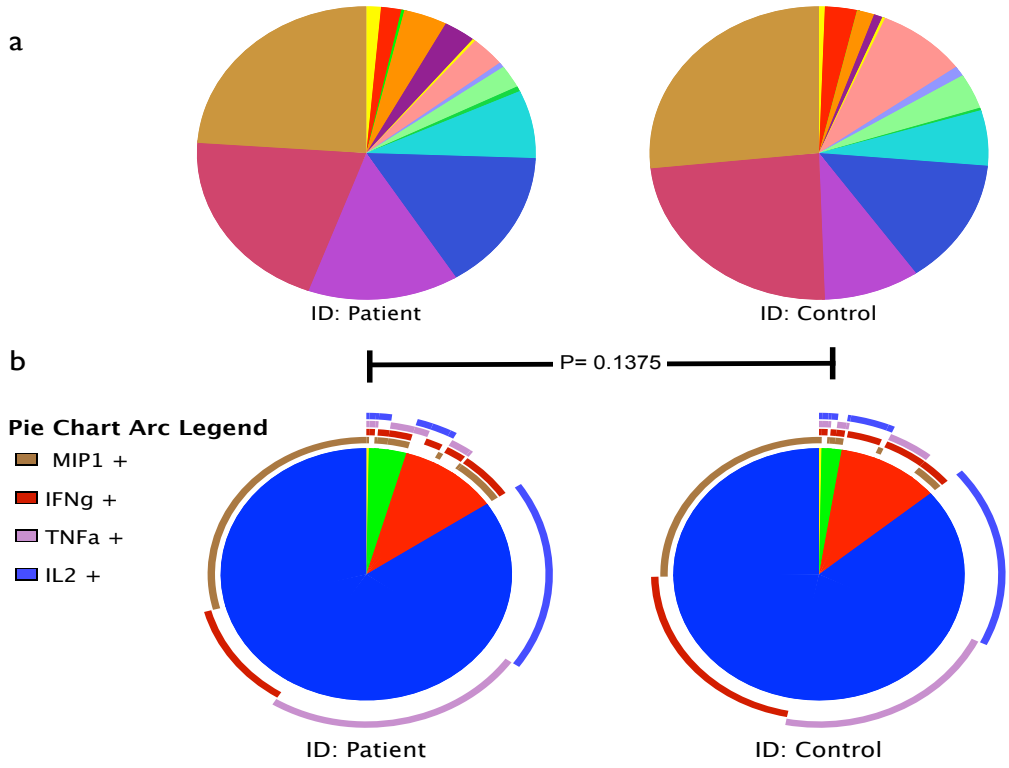


Figure 5.11: Comparison of the pattern of polyfunctionality of CMV specific CD8+ T cells in CAD patients and healthy donors.

(a) Pie charts represent the entire pattern of multifunctional CD8+ T cells among patients and controls groups. Each slice in the pie charts represents one of the fifteen possible combinations of the four functions (MIP1β, IFNγ, TNFα, and IL-2). Therefore, colours of slices match the colours that are given to the square below the bars in Figure 5.10. The pie charts (**b**) are duplicates of the pie charts in (**a**), however the fifteen functional combination sectors were replaced by the main four groups i.e CD8+ T cells having one function (1+), two functions (2+), three functions (3+), and four functions (4+). Therefore, there are only four different colours, which were given the same colours as corresponding horizontal bars in Figure 5.10. For clarification purposes, arcs were added to each pie chart, where each arc symbolizes a single function. The overlap between arcs defines the pattern of polyfunctionality.

5.4.10 Similarity between the patterns of polyfunctionality of CD4+ T cell responses and CD8+ T cell responses to CMV antigen.

Regardless of the magnitude of the responses, the CD4+ T cell responses to CMV antigens showed a very similar pattern to those of CD8+ T cells. In both situations the response that predominates is with cells that express only one function. For instance MIP1β and IFNγ single responding cells compose just less than the half of all responses. Meanwhile, the summation of polyfunctional cells (two or more functions) constitutes less than 30% of the responding cells (Figure 5.12).

Comparing side-by-side CD4+ T cells response of CAD patient and controls showed no statistical difference between any compared pairs of data, see integrated table in Figure 5.12. Furthermore comparing the two groups using permutation analysis confirmed no dramatic difference in the quality of the CD4+ T cell response ($p=0.24$), where the polyfunctional cells represent approximately 25% of responding cells in both patient and control groups, see pie chart Figure 5.12.

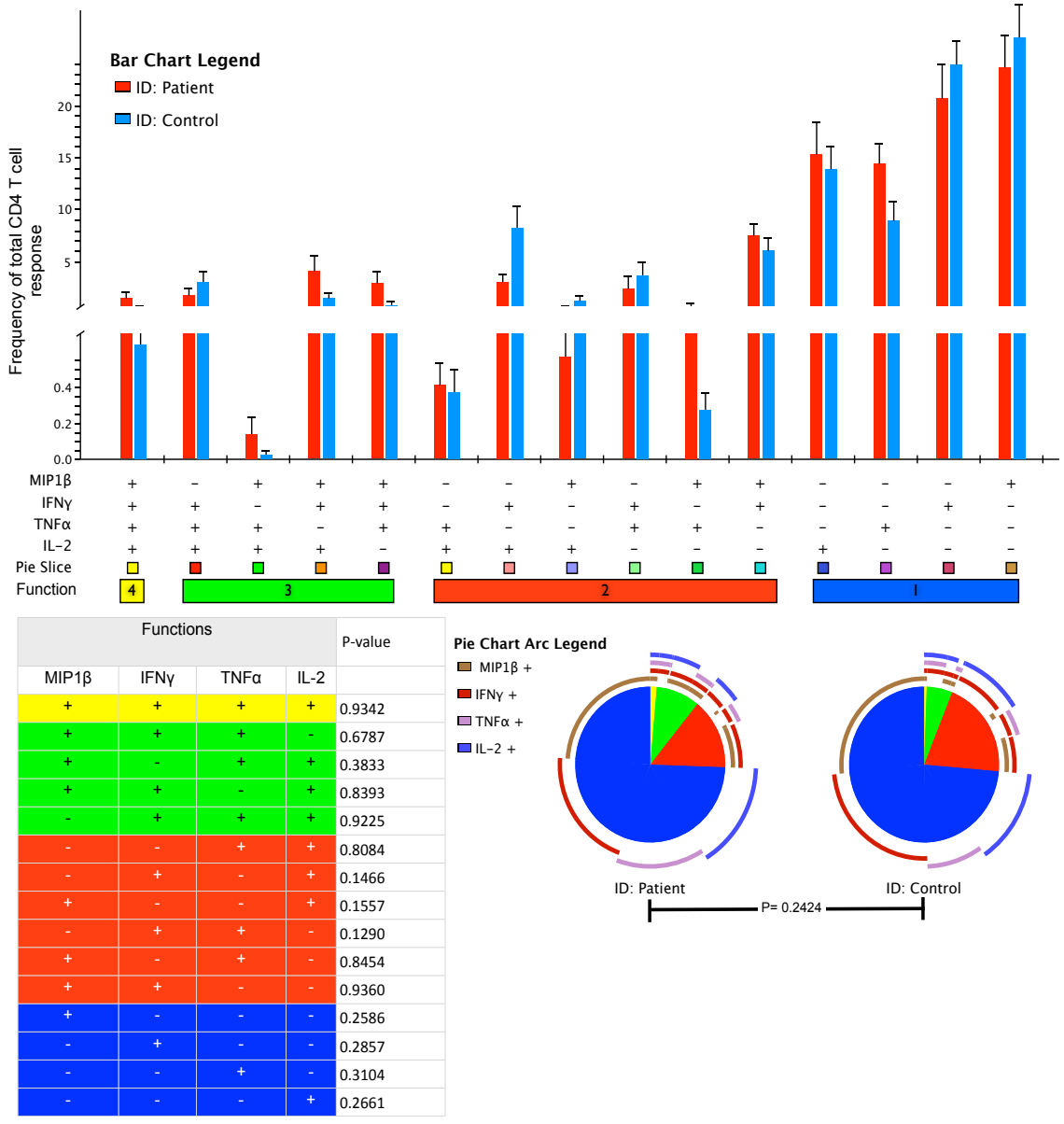


Figure 5.12: Comparison of the quality of CD4+ T cells responses to CMV antigens in CAD patient and control groups.

As in Figure 5.10 the bar chart compares the 15 possible function combinations in patients and controls. The table shows the statistical differences (P value) of this comparison based on the Mann-Whitney test. The pie charts demonstrate the pattern of the whole difference between the four major function categories.

5.4.11 Patients with stable post myocardial infarction preferentially maintain highly functional CMV-specific T cells compared to patients with Acute Myocardial Infarction.

Clinically, the patient cohort comprises of two categories of coronary artery disease patients, i.e. AMI and PMI. Consequently, it was investigated whether the pattern of CMV specific T cell polyfunctionality was related to the disease categories.

The initial observation was that the permutation analysis of the CMV specific CD4+ T cell functional pattern in the PMI patient group preserved high functionality compared to both AMI patients (P= 0.001) and the control group (P= 0.005), see pie chart in Figure 5.13. In this context, the difference between PMI and AMI seems to be a result of differences in cells with any two and any three functional combinations (red and green sectors respectively of the pie charts in Figure 5.13). Furthermore, this difference was particularly significant when comparison was made at the level of MIP1β+ IFNγ- TNFα+ and IL-2+ combinations (one-way ANOVA test, P< 0.05). In addition, there were significant differences in MIP1β-, IFNγ-, TNFα+, IL-2+ CD4+ T cells and MIP1β+, IFNγ-, TNFα- IL-2+ CD4+ T cells combinations (P<0.01 and P<0.05 respectively). In contrast, the difference in CD4+ T cell quality between the PMI group and the control group is mainly due to monofunctional cells, for instance, while there were more IL-2 and TNFα single producer cells in the PMI group, the control group had more MIP1β and IFNγ single producer cells. Besides, there is a slightly higher preponderance in three functional combinations and statistically significant differences in MIP1β+, IFNγ-, TNFα- IL-2+ CD4 T cells (P<0.05).

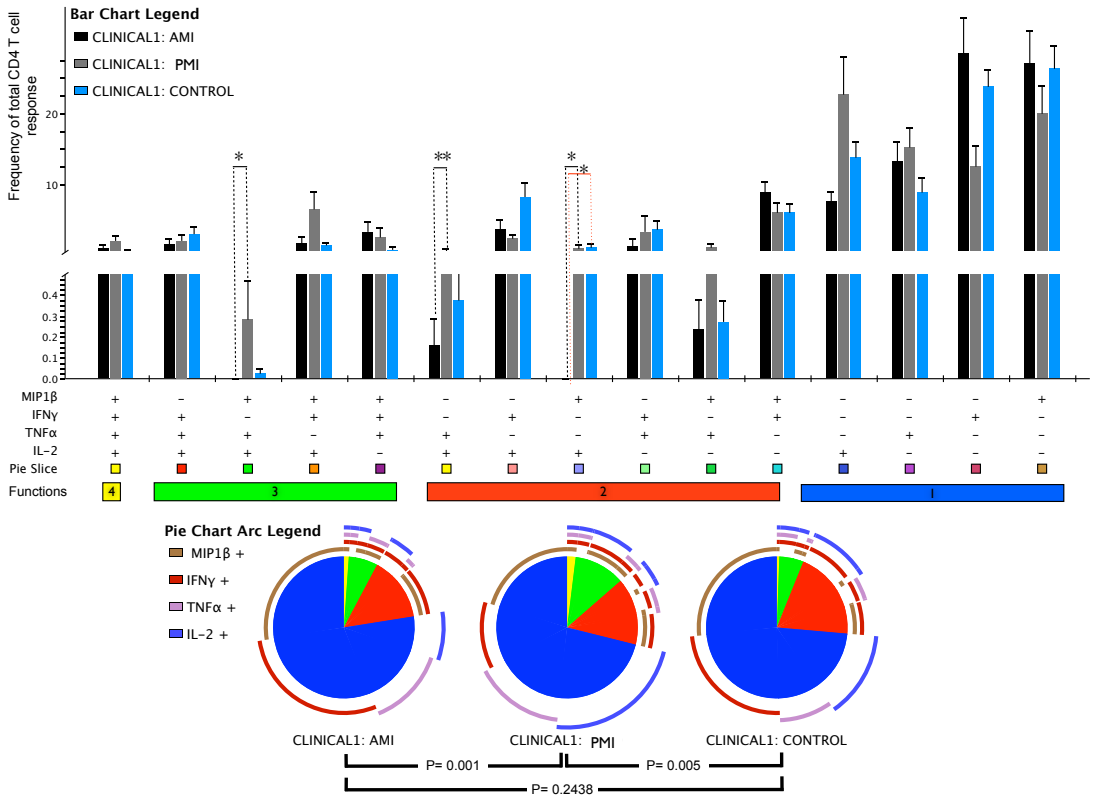


Figure 5.13: Comparing the quality of CD4 + T cell response to CMV antigens in CAD patient sub-groups (AMI and PMI) and the control group.

The histogram compares 15 functional combinations of CMV specific CD4+ T cells in acute myocardial infarction (AMI) and patients with stable post myocardial infarction (PMI) and healthy controls (black and grey and blue bars respectively). Asterisks at the top of bars refer to the significance of the difference between the groups (based on one-way ANOVA test and Dunn's Multiple Comparison post Test). For instance one asterisk means $P< 0.05$, while two and three asterisks symbolize $P<0.01$ and $P<0.001$ respectively. The pie charts demonstrate the pattern of polyfunctionality. The differences in the pattern were tested by permutation and are presented as P values under the charts.

Contrasting with CD4+ T cells, permutation analysis of the CMV specific CD8+ T cell features between PMI, AMI and the control group showed no substantial variances (see pie charts in Figure 5.14). Nevertheless, the comparison at the level of paired groups revealed several statistical differences; see histogram Figure 5.14. An example of that is remarkably high frequency of four functions positive CD8+ T cells in PMI comparing to the control group ($P<0.05$). Besides, PMI showed a greater level of MIP1β+ IFNγ+ TNFα- IL-2+ CD8+ T cells comparing to both AMI and the control group.

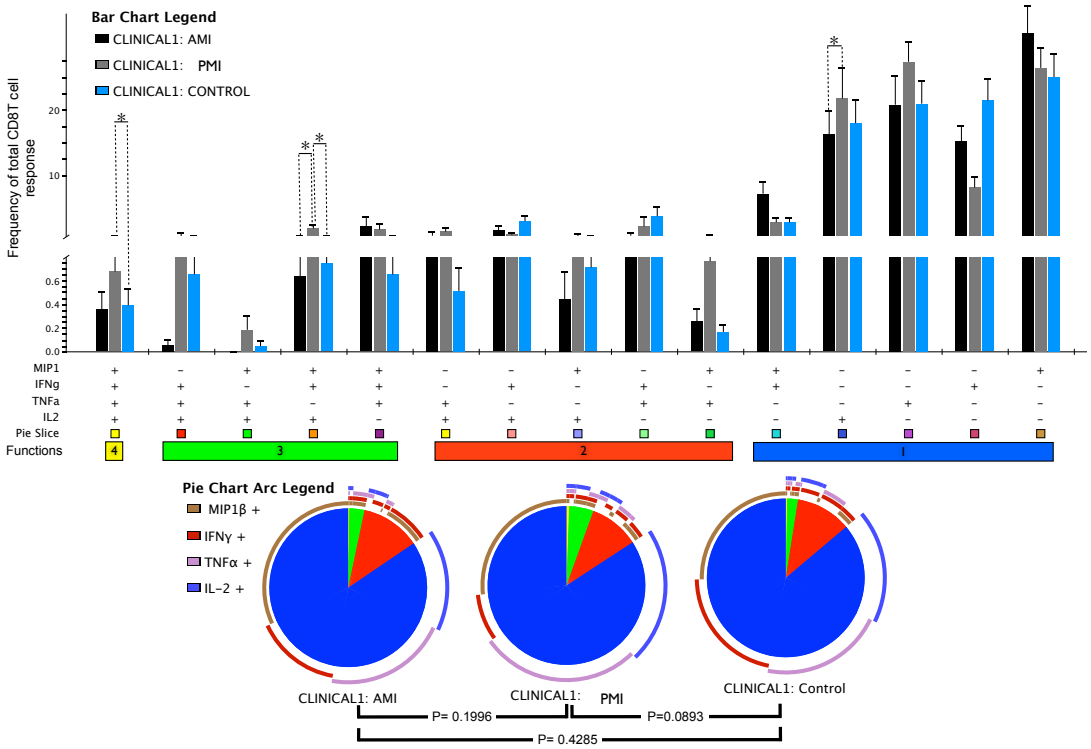


Figure 5.14: Comparing the quality of CD8+ T cell responses to CMV antigens in IHD patient sub-groups (AMI and PMI).

Similar to Figure 4.13, the histogram compares 15 functional combinations of CMV specific CD8+ T cells in AMI, PMI, and control groups. The significant differences are indicated with asterisks. The pie charts demonstrate the pattern of the whole differences between the four major functional categories with a permutation test.

5.4.12 In addition to CMV seropositivity, coronary heart disease *per se* might have an impact on T cell quality.

Having observed differences in the patterns of functionality in response to CMV antigens between patients and controls, particularly in CD4+ T cells, it was hypothesized that, regardless of the CMV status, these differences in T cell quality might be a consequence of coronary heart disease itself and not the effect of CMV carriage. Aiming to prove or disprove that it was investigated if the same differences could be identified between diseased and healthy control groups who were CMV seronegative. This time a mitogen (CON A) was used to trigger T cell responses rather than CMV antigens.

The pie charts in Figure 4.15 summarize the permutation analysis of CD4+ and CD8+ T cell responses to mitogen in AMI, PMI, and control groups. Interestingly, there were significant differences in CD4+ and CD8+ T cell responses between PMI, AMI and control groups. However, these differences related to single functional cells, such as only IFNγ+ and IL-2+ producing cells (represented as red and blue arcs in the pie charts respectively) (Figure 5.15) rather than differences in the polyfunctional cells. For instance the red, green, and yellow slices, which symbolize 2+, 3+, and 4+ functional combinations and their corresponding overlapped arcs are very similar in all groups.

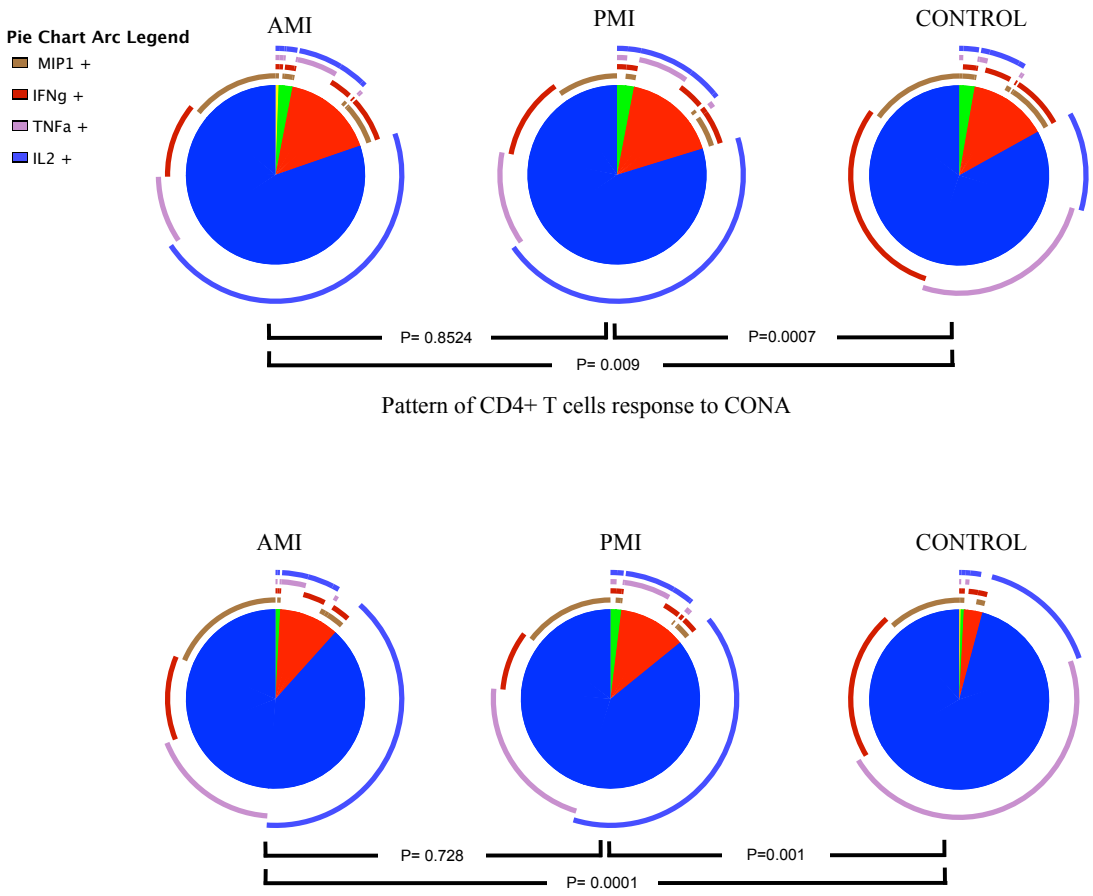


Figure 5.15: Comparison of the quality of T cell responses to mitogen in CAD patient sub-groups and the control group.

The pattern of T cell responses to Mitogen (CON A) in CMV seronegative patient and control groups. The top row of pie charts shows the differences in CD4+ T cell responses while the bottom row demonstrates CD8+ T cell responses in the same groups. The blue sector of the pie indicates the percentage of single function cells in the total response while the red, green, and yellow portions represent the polyfunctional cell percentages. Each pie chart arc symbolizes a single function and the overlap between arcs determines the pattern of polyfunctionality. A permutation test was used to show the differences between these groups and differences are presented as P values under the charts.

5.4.13 Polyfunctional CMV specific T cells produce more cytokine on a per cell basis.

Having shown a high polyfunctionality of CMV specific T cells in the PMI group compared to both the AMI and control groups, it was investigated if there was any association between the degree of functionality and the amount of the chemokine/ cytokine produced per cell. The median fluorescence intensity (MFI) of each of the four markers within the fifteen functional combinations was calculated (Figure 5.16). Here it is shown that there was a direct relationship between the polyfunctionality and the expression levels of cytokines. In other words, the higher the functionality the higher the chemokine/cytokine levels. For instance, the MFI of IFNγ in 4+ polyfunctional CMV specific CD4+ and CD8+ T cells was approximately 3-fold and 2.5-fold higher, respectively, compared to mono-functional CMV specific CD4+ and CD8+ T cells. A similar trend was seen in all other chemokine/cytokines (Figure 5.17) the only exception was the IL-2 secretion level, which was remarkably high in both CD4+ and CD8+ T cells with two concurrent functions compared to those displaying three or four functions.

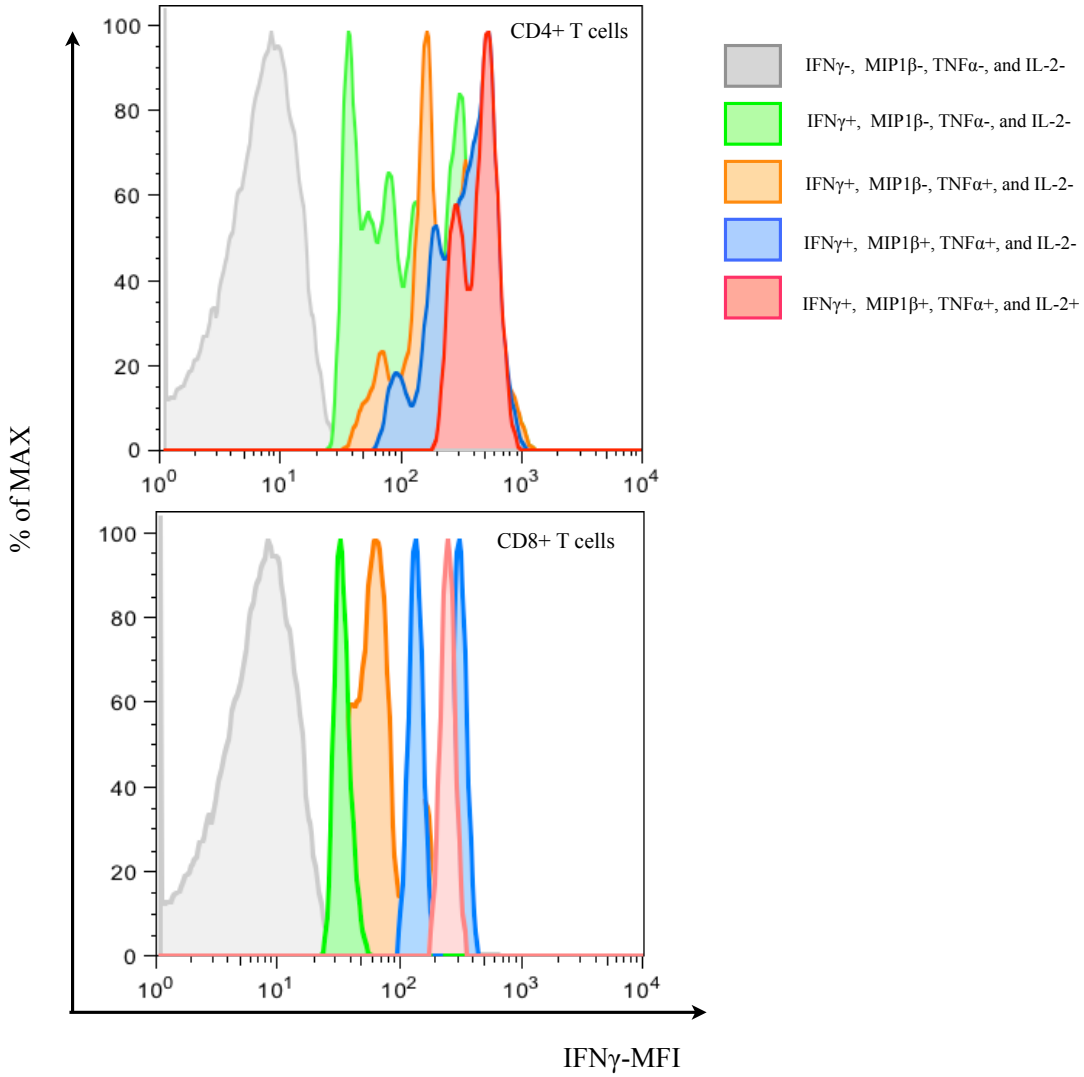


Figure 5.16: IFNγ median fluorescence intensity (MFI) is higher in polyfunctional CMV specific T cells.

A representative example of the IFNγ-MFI overlay of cells producing different cytokine combinations in CD4+ T cells (upper plot) and CD8+ T cells (lower plot). The IFNγ-MFI of each functional combination was colour coded and represented as a histogram. (See figure label)

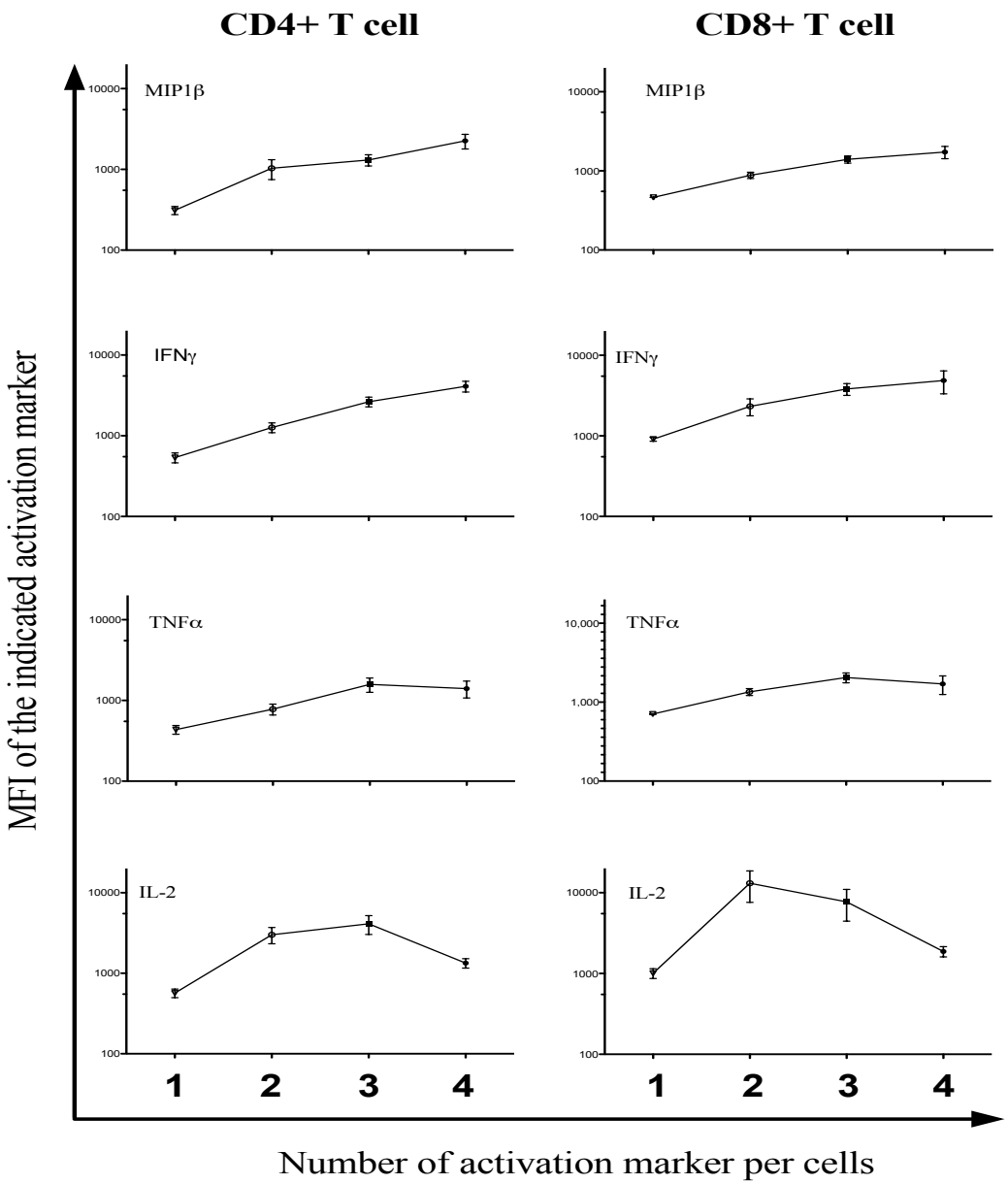


Figure 5.17: CMV specific polyfunctional T cells express higher levels of cytokines than CMV specific monofunctional cells.

Each graph shows median fluorescence intensity (MFI +/- SEM) of MIP1β, IFNγ, TNFα, and IL-2 in CMV specific T cells (CD4+ and CD8+; left and right column of plots respectively). The Y-axis indicates MFI levels and the X-axis shows T cells expressing one, two, three or four cytokines.

5.5 Discussion

The purpose of this project was to investigate the relationship between CMV specific T cell responses and coronary heart disease. Thus the polyfunctionality of CMV specific T cells in CAD patients was compared to that in healthy controls. The objective was to measure a panel of chemokine/cytokines including; CD107a, MIP1β, IFNγ, TNFα, and IL-2 utilizing flow cytometry and intracellular cytokine staining (ICS). It is worthy to mention that, there is no consensus about which markers should be used for these purposes and justifications for selecting this panel of markers that were used in this project is discussed elsewhere (see section 7.1.3). Indeed, measuring such a relatively large panel of cytokines technically necessitates several optimization steps before commencing actual experiments (Gauduin *et al.*, 2004, Lamoreaux *et al.*, 2006, Horton *et al.*, 2007, Nomura *et al.*, 2008).

5.5.1 The importance of optimization steps

Taking into consideration that the accuracy of the ICS assay is highly dependent on the condition of the PBMC after being thawed, two strategies were used to improve PBMC condition. The first strategy was to add DNase to the washing media during media exchange steps and during resting periods as well as during the stimulation step of ICS. This step was based on previous studies which shown that using DNase during washing steps remarkably decreased PBMC stickiness (Darzynkiewicz *et al.*, 1994, Lamoreaux *et al.*, 2006). The second strategy was to incorporate a resting period for PBMC before commencing the stimulation and ICS steps.

Since the use of DNase was novel in this laboratory, it was essential to confirm that the proposed DNase concentration did not interfere with the ICS assay. Therefore a set of optimization experiments was performed. The first observation from these experiments was an improvement in the yield of PBMC, which was reflected by increased numbers of cells harvested from frozen samples washed with DNase. This illustrates the benefit of minimizing the PBMC loss caused by clumping of cells together, and thus physically removing them during sample filtration. It is known that the freeze-thaw process is very stressful to PBMC and can lead to damage of cells and release of DNA, which can cause adherence of cells and occurrence of PBMC stickiness phenomena.

The second observation was that incorporation of a PBMC resting period and including DNase in the ICS protocol led to a remarkable reduction in background staining for cytokines in the mock samples. This was of particular importance because the response of CMV stimulated samples was determined by subtraction of the background staining in the mock sample. In other words, this means an increase in the assay specificity and sensitivity. A possible explanation might be that the resting of PBMC for a long enough period in the presence of DNase gives more time to destroy any potential apoptotic cells and eliminate any released DNA by DNase. Thus, this will reduce the nonspecific binding of monoclonal antibody to DNA during the ICS step.

5.5.2 Determining which functions to measure of the T cell response

Before discussing further, it is essential here to clarify exactly the meaning of some terms. For instant *single cytokine producing cell* or *single function cells* refer to cells that produce or express only one cytokine or function that was measured in this project. For

example the frequency of IFNγ single producing cells means the frequency of cells in the population that only produce IFNγ but not other measured markers. Nevertheless, it should be emphesised that these cells might be still able to produce other cytokines that are not included in the measured panel such as IL-17, and IL-4. Meanwhile, the frequency of IFNγ producing cells (without prefix 'Single' or 'Mono') means the frequency of all those cell producing IFNγ regardless if they express other functions or not. 'Polyfunctional' or 'multifunctional' will be used to describe cells that can produce more than a single cytokine simultaneously.

Stereotypically, the magnitude of the CMV specific T cell response is defined by measuring the frequency of antigen-specific T cells based on expression of certain specific effector function such as IFNγ and TNFα productions (Khan *et al.*, 2004, Sester *et al.*, 2005, Sund *et al.*, 2010). Nonetheless, measuring of a single parameter independent of other markers might not always precisely describe the magnitude of the T cell response (Ouyang *et al.*, 2003b, Darrah *et al.*, 2007, Nebbia *et al.*, 2008). Particularly if we take into consideration that T cell response to a stimulus is not only restricted to effector function but also involves the ability to proliferate or induce proliferation and modulation of other cells of the immune system so that any proper description of T cell function should cover these aspects.

Therefore aiming to define an appropriate measure of T cell response quality, experiments were designed to involve a panel of different activation markers. However, due to limitations in the ability of flow cytometry to detect only eight colours, in this

thesis measuring the components CD107a, MIP1β, IFNγ, TNFα, and IL-2 was thought to give a comprehensive picture of the quality of the antigen-specific T cell response.

Returning to the optimization aspect of this project and as mentioned in the Aims, one of the targets was to characterise the cytotoxic activity of CMV specific T cells. Therefore the CD107 mobilization assay was adopted. This assay works by detecting the transient surface expression of CD107a or CD107b (LAMP-1/LAMP-2 respectively) that takes place during T cell degranulation following antigenic stimulation. This assay involves *ex vivo* addition of a protein transport inhibitor to stimulated lymphocytes. This leads to blocking of the intracellular protein transport and accordingly accumulation of cytokine inside the cells and also prevents the quick reinternalization of CD107 molecules after degranulation. Therefore, it will enhance the detectability of both cytokine-producing cells and expression of CD107 by flow cytometry. Brefeldin A (BFA) and monensin are the cytokine secretion inhibitors that are most commonly used in ICS. However, they have been found to have different effects on ICS, particularly the duration of incubation and cytokines that are required to be detected (Nomura *et al.*, 2008). For instance, the viability of monensin treated cells is significantly decreased when exceeding 18 hours incubation compared with Brefeldin A, which is less toxic to the cells (O'Neil-Andersen and Lawrence, 2002). Nevertheless, monensin must be included in the protocol as it is required to prevent acidification of lysosomes and endosomes and is consequently preferable for inhibition of CD107 reuptake than Brefeldin A. Taking into consideration the advantages and disadvantages of both reagents the decision was made to use both of them concurrently. The question, which needed to be asked here, is that, is it possible to use both inhibitors simultaneously without affecting the accuracy of the assay? To

answer this question, levels of CD107a and IFNγ were measured in the presence and absence of monensin, (see Section 5.4.3.2). It was found that stimulating PBMC in the presence of monensin for approximately 12 hours improved the CD107 detection threshold without affecting the ICS assay.

5.5.3 Justification for using a special statistical analysis software

Aiming to measure the full complexity of the T cell response, an array of Boolean gates of response markers, which include MIP1β, IFNγ, TNFα, and IL-2, was generated. The result of this was 15 possible combinations of these functions. For appropriate analysis of such polychromatic flow cytometry data' special programs called PESTLE and SPICE v5 (Simplified Presentation of Incredibly Complex Evaluations, Version 5) were used (Roederer, 2011).

Statistically, since the data was continuous, unpaired and does not follow Gaussian distribution, then all statistical tests used were nonparametric; this included the Mann Whitney test (in case of comparing two independent variables) and the Kruskal-Wallis test (variant of One-Way ANOVA test) followed by Dunn's post-test in cases where more than two variables were compared.

One of the simplest ways to determine differences in the whole pattern of T cell functionality is to represent data as pie charts, followed by comparing each portion of certain pie charts to its corresponding portion in other pie charts. Nevertheless comparison of distributions from individual categories in these complex datasets might lead to Type 1 statistical errors. In other words it can lead to mistaken identification of a

difference as being statistically significant. This comes from the large number of comparison all of the distributions simultaneously. To overcome this a permutation test was adapted that can be done by the SPICE program. Basically SPICE compares the size of each category (each "slice") between the two distributions. It sums up the differences over all categories (all slices) to determine the overall difference. This overall difference is used in the permutation test. Differences were considered statistically significant when $p<0.05$.

The current study showed that the percent T cells expressing a single function independently of other functions was significant higher in the patient group compared to controls. This was particularly relevant when considering IFNγ or TNFα positive CMV specific CD8+ T cells, see (Section 5.2.5). However, the pattern of responding cells was approximately similar in both the patient and control groups. For instance, the highest frequency of responding CMV reacting cells usually dominated by those cells producing MIP1β followed by TNFα or IL-2 and then IFNγ. This finding seems to be consistent with the earlier observation, which showed correlation between high inflammatory cytokine levels and coronary artery disease (Caligiuri *et al.*, 1998, Jonasson *et al.*, 2002, Simanek *et al.*, 2011). Furthermore it might suggest that repeated reactivation of CMV specific CD8+ T cells can act as a source of inflammatory cytokines.

Interestingly, examining the T cell response of each donor individually demonstrated that no functional marker was consistently expressed in all responding cells which might suggest that it is not appropriate to rely on measuring each function independently of other to determine the response. This is supported by the same observation in many HIV studies that confirm that measuring individual effector cytokines may be an

inefficient indicator of the T cell response (Betts *et al.*, 2006, Kim *et al.*, 2009b). Therefore, we carried out Boolean gating of the four cytokines to compare all possible combinations of the four functions. In this regard, the current study indicates that the majority of T cell responses, irrespective of the donor status (patient or healthy), are characterized by low functionality. Most responding cells are restricted in expressing only one chemokine or cytokine, and only less than 30% of the response can be considered as multifunctional with very few donors having T cells expressing four functions concurrently.

Considering the patient cohort consisted of two substantially different groups in the spectrum of coronary heart disease; namely AMI and PMI then it was more appropriate to investigate them as different groups rather than as a single combined group. Interestingly, subdividing patients into AMI and PMI revealed several significant differences in their T cell response quality. An example of this is the high frequency of 2+ and 3+ functional CMV specific CD4+ T cells and 4+ functional CMV specific CD8+ T cells in PMI compared to AMI. Furthermore, the permutation analysis showed that PMI preferentially maintains highly functional CMV-specific T cells, particularly CD4+ T cells, compared to patients with AMI and to controls (Section 5.4.11). For instance, comparing PMI to AMI showed that in PMI there were higher frequencies of CD4+ T cell populations that have one of the following chemokine/ cytokine profiles (MIP1β+, IFNγ-, TNFα-, and IL-2+), (MIP1β-, IFNγ-, TNFα+, and IL-2+) or (MIP1β+, IFNγ-, TNFα+, and IL-2+).

Interestingly, all of these profiles are characterized by the absence of IFNγ and the presence of IL-2 plus any other functions. This might suggest the importance of a

positive IL-2 and negative IFNγ T cell profile as a prognostic factor in coronary artery disease in CMV carriers. Supporting this view are the studies by Sinclair et al (Sinclair *et al.*, 2006) who observed significantly lower numbers of CMV-specific T cells that express IL-2 in subjects with active CMV retinitis. Furthermore Gattinoni and colleagues (Gattinoni *et al.*, 2005) demonstrated that the frequency of effector T cells, characterised by strong cytotoxic function and IFNγ production, are paradoxically correlated with the control of cancer growth in a cancer immunotherapy model. Additionally the results of this study is consists with the model of Pantaleo et al (Pantaleo and Harari, 2006, Harari *et al.*, 2009) that proposed that IL-2 production in the absence of IFNγ by antigen specific T cells is a signature for viral clearance. Meanwhile dominance of IFNγ in the absence of IL-2 is associated with chronic non-controlled disease whereas synchronized secretion of both of them by the same cell is seen in chronic controlled infections (Figure 5.18).

Contradictory to the findings of the current study, Casazza et al showed that the salient features of virus-specific CD4+ T cells in chronic CMV infection characterise by the simultaneous production of MIP1β, TNFα, and IFNγ in the absence of IL-2 (Casazza *et al.*, 2006). Nonetheless, in their study day used only eight PP65 CMV epitopes. Meanwhile in the current experiments comprehensive CMV peptide pools were used for this purpose. These pools covered the entire amino acid sequences of the HCMV pp65, major immediate early (IE-1) proteins and DYS/VTE. Therefore the Casazza group work might reflect only the functional profile for CMV pp65 specific CD4+ T cells.

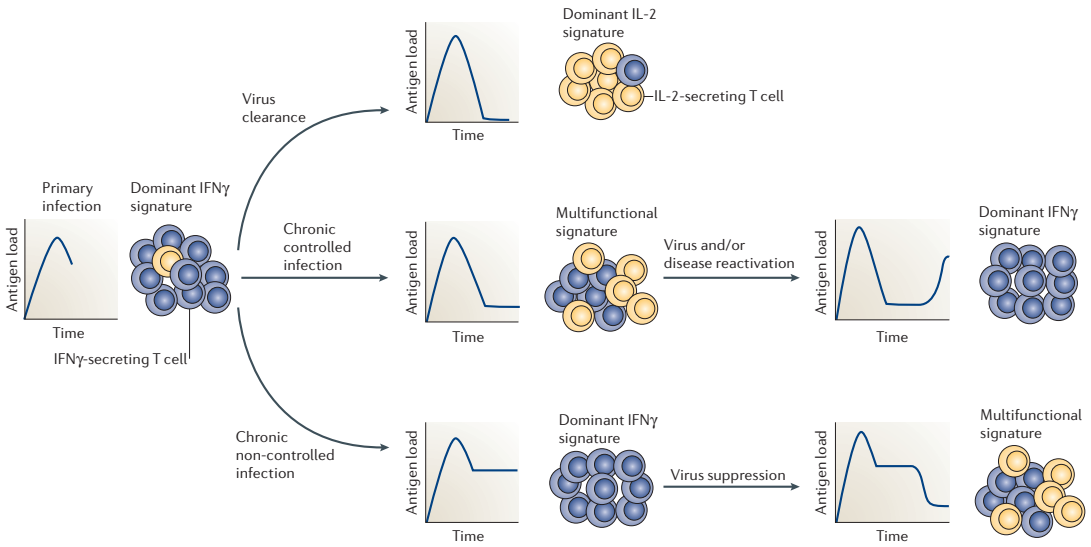


Figure 5.18: Pantaleo and Harari model for functional signatures (IFNγ and IL-2) of virus-specific CD4+ and CD8+ T cells to clinical monitoring of virus-associated disease

This Figure illustrates the possible fate of the cytokine profile of T cells during infection. The primary viral infection is usually dominated by an IFNγ signature of CD4+ and CD8+ T cells (depicted in blue) with a small number of IL-2 secreting cells (depicted in yellow). After primary infection, there are three possible outcomes: virus elimination associated with a dominant IL-2 signature of the T cell response, establishment of chronic controlled infection associated with a multifunctional T cell response, and establishment of chronic non-controlled infection associated with the persistence of a dominant IFNγ signature. In the case of chronic controlled infection, reactivation of viral replication will be marked by a rapid shift from a multifunctional to a dominant IFNγ signature. In the case of non-controlled infection, suppression of viral replication for example by treatment will be marked by a shift from a dominant IFNγ to a multifunctional signature. Figure adopted from (Pantaleo and Harari, 2006)

Having observed this shifting in T cells response post myocardial infarction towards polyfunctionality raised a question about the role of coronary artery disease in modelling patterns of T cell response. One might argue that changes in CMV specific T cell functionality is a part of global phenomenon in CAD affecting the whole memory T cell pool. Consequently, one can propose that ischaemic heart disease *per se* might cause changes in the whole T cell response including the CMV specific one. To address this possibility we investigate the T cell response of CMV seronegative cohorts to mitogen (Section 5.4.12). In this context, it was shown that the differences in cell quality among the patient subgroups and between them and the control group were mainly due to differences in magnitude of monofunctional cells and not as a consequence of shifting to polyfunctionality.

Rationally we expected that the capability of one cell to secrete numerous cytokines simultaneously might negatively affect the amount of these cytokines expressed per cell. Interestingly, opposite to expectation, CMV specific polyfunctional T cells were found to be capable of expressing larger quantities of cytokines compared to CMV specific monofunctional cells. For example, amounts of IFNγ and TNFα produced by each polyfunctional cell were about 2.5 to 3 times higher than the levels of these cytokines in monofunctional cells. In other words, the level of each cytokine positively correlated to the number of functions expressed per cell. The only exception to this rule was the level of IL-2, which was secreted more by double functional than polyfunctional cells. This might be explained by the role of IL-2 being to maintaining T cell proliferation in early immune responses to antigen. This again supports the notion that polyfunctional cells

are more efficient effector cells than the monofunctional cells. Therefore, one can propose that even small differences in the frequency of polyfunctional cells might be biologically important and could have a major influence on the quality of antigen specific immune responses.

6 Prospective Study of Immunity to Varicella Zoster Virus During Treatment For Acute Lymphoblastic Leukaemia (All) In Children

6.1 Background

Varicella zoster virus (VZV) is a cytopathic human herpes virus that causes chickenpox (varicella) upon primary infection. This is followed by persistent infection in the body in a dormant state, termed as latency, from which the virus may reactivate to cause shingles (zoster) (Arvin, 2001). More severe disease symptoms occur in persons with a weak immune system such as children with immunodeficiency, pregnant women and elderly persons, suggesting that the virus is successfully kept in check by immune mechanisms in healthy persons with normal immune function.

In a recent epidemiological study in the UK and Ireland 112, children have been identified with severe complications of chickenpox and six deaths, over a 13-month period from November 2002 (0.82/100,000 children/year) (Cameron *et al.*, 2007). Four deaths were in children with underlying medical conditions although not ones usually associated with high varicella mortality. Furthermore, the development of chickenpox in immunocompromised children was found to be not only associated with considerable morbidity but also with increased mortality rate. For instance, the overall mortality rate from varicella in children with cancer has been reported in the late 1980's to be as high as 7% (Feldman *et al.*, 1975, Feldman and Lott, 1987). However, the subsequent introduction of therapeutic acyclovir shortens the cutaneous course of the infection and reduces the risk of dissemination and a more serious illness (Feldman and Lott, 1987, Nyerges *et al.*, 1987).

It was previous practice on the Regional Oncology Unit at Alder Hey to give prophylactic oral acyclovir to all immunocompromised patients following contact with VZV. In 2003, the practice was changed in accordance with National Guidelines (RCPCH, 2002) and prophylaxes only were given to patients without varicella zoster immunoglobulin G (VZV IgG) detected at diagnosis.

Taking into consideration that at least 70% of all patients in the Oncology Unit at Alder Hey had VZV IgG detected at diagnosis of their malignancy suggesting previous infection with chickenpox, led to a reduction in the prescription of prophylactic acyclovir and a considerable cost saving. However, between July 2001 and July 2007, 40 patients with a malignancy in the Oncology Unit at Alder Hey were diagnosed with chickenpox. Nine of these 40 patients were found to have VZV IgG detected at diagnosis of their malignancy (Manley *et al.*, 2008). The primary diagnosis of all nine patients was acute lymphoblastic leukaemia (ALL) and all developed chickenpox between November 2004 and July 2007. None of these patients had clinically dermatomal shingles and no patient received prophylactic acyclovir, as their chickenpox occurred following the change in practice in 2003.

Eight of these nine patients had their VZV IgG rechecked either at the time or after the episode of chickenpox. Five of the patients showed loss of VZV IgG at the start of their chickenpox and three patients showed a further decline in VZV IgG at some time after the event. All these cases of chickenpox occurred in the second or third year of chemotherapy.

Indeed, the development of chickenpox in immunocompromised children, who were thought to have adequate protection, is a worrying occurrence and needs to be investigated.

Usually, protection against herpes zoster is correlated with the antibody-mediated immune response through measurement of VZV-specific antibody by ELISA. However, in view of the importance of T cells in recognition of intracellular viral antigens, it has been argued that this form of immune response is of equal or even greater importance than antibody responses (Heininger and Seward, 2006).

This is supported by the observation that the decline in the T cell response coincides with higher levels of viral reactivation (Asanuma *et al.*, 2000). Therefore, the current study proposes the investigation of antibody-mediated responses against VZV alongside T cell anti-viral immune responses.

Research question

To determine whether there is loss of immunity to varicella in children with ALL treated with the current national protocol ALL 2003, as a result of the immunosuppressive therapy by studying VZV-specific B and T cell immunity.

6.2 Aims

To determine the importance of B or T cell mediated immunity to protect against VZV infection and reactivation during chemotherapy in patients with ALL. Furthermore, to evaluate whether the loss of VZV-specific B and/or T cell mediated immunity might be utilized as a risk predictor for VZV reactivation in patients who were considered to be protected. Therefore, the following two steps were carried out:

1. Monitoring the change in the levels of VZV IgG as indicator of B cell function.
2. Assessing any changes in the VZV-specific T cell immune responses through measuring cytokine profiles.

6.3 Plan of Investigation

6.3.1 Study population

6.3.1.1 Control group

Since having healthy children, as a control group was ethically unattainable, healthy adult donors were used. The main purpose of that was to optimize different assays used in this project. 45 donors aged from 18-40 years were recruited for this purpose. Therefore, in this chapter whenever 'adult healthy controls' are mentioned it refers to this group unless otherwise mentioned.

6.3.1.2 Patient group and sampling time point

This work involves children diagnosed with acute lymphocytic leukaemia (ALL) and treated according to the ALL 2003 protocol. Both newly diagnosed patients and patients already undergoing chemotherapy were included. Table 6-1 summarises the patient group characteristics.

The number of blood samples that were taken from each patient depended on two main factors. Firstly, the stage of treatment at which any patient enrolled into the study, the second is the type of the chemotherapy regimen. For instance, at diagnosis, a patient would receive either Regimen A or Regimen B, dependent on age and presenting white cell count. However, for a variety of reasons, a patient might be transferred at any point to Regimen C. Furthermore, the length of each regimen is also dependent on the gender of the patient. Hence, fewer samples were taken from girls as they complete treatment at week 112 in Regimen A, week 114 in Regimen B and week 118 in Regimen C. In contrast, boys will complete treatment at week 164 in Regimen A, week 166 in Regimen B and week 170 in Regimen C. Therefore, the goal was to collect five samples from girls and seven samples from boys.

Table 6-1: Age and gender distribution of the leukaemic children group

Age at study entry	< 3 years		3 to 5 years		> 5 years		Total	
Gender	Male	Female	Male	Female	Male	Female	Male	Female
Number	5	5	10	11	15	28	44	30
Total	10		21		43		74	

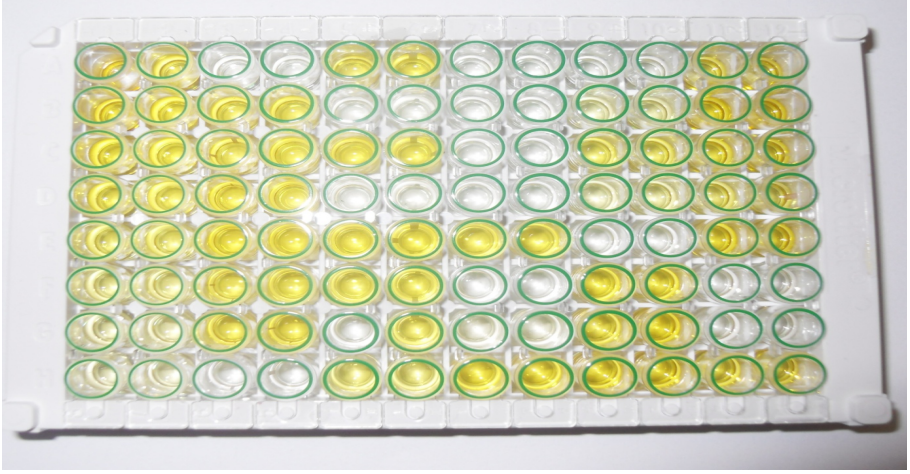
6.4 Statistical analysis

Unless otherwise mentioned all data were presented as median with interquartile range and Mann Whitney test was applied with 95% confidence intervals to test differences between different donor groups. All P values were two-tailed and modified for multiple comparisons according to the Holm-Bonferroni correction. Receiver-Operator Characteristic (ROC) curve, was used to determine the cut off point of positivity and negativity in ICS assay based on the trade-off between the high sensitivity and high specificity.

6.5 Results

6.5.1 Testing the study cohort for VZV status

To address the aim of monitoring VZV-specific B cell changes, all serum samples from patient groups were sent to the Department of Clinical Microbiology, Aintree Hospital Foundation NHS Trust for VZV IgG determination where 1 ml of serum/plasma was examined by ELISA using a Diamedix Immunosimplicity (Is)-VZV IgG test kit (IVAX Diagnostics, Inc., Miami, FL). Index values were recorded to represent a quantitative measure of VZV IgG antibody levels, with a level of less than 16 EU/ml indicating no immunity, level 16-19 EU/ml considered as equivocal and ≥20 signifying immunity to varicella. Regarding the healthy adult control group, 45 consenting volunteers were recruited to donate 20 ml of blood. After separating the PBMC buffy layer, plasma was used for quantitative VZV IgG ELISA using a Diamedix Immunosimplicity (Is)-VZV IgG test system (Cat. No. 720-380; 96 tests). An illustrative example of the ELISA test from healthy donors is shown in Figure 6.1, for more details see section 2.3.1.3.



	1	2	3	4	5	6	7	8	9	10	11	12
A	1.504	2.012	3.05	2.95	59.35	69.15	3	2.95	3.15	3.4	45.9	50.2
B	1.771	1.708	60.5	63.4	2.7	3.25	3.55	3.3	16.25	18.9	82.05	83.25
C	1.517	1.426	55.25	65	76.7	70.35	2.65	2.6	49.1	43.5	58	60.65
D	1.193	1.152	68.25	64.5	3.5	3.75	2.7	2.65	14.7	21	35.3	35.35
E	0.831	0.844	80.7	78	75.8	73.8	59.2	63.7	2.75	2.75	78.7	70.1
F	0.531	0.556	54.85	60.8	53.8	58.8	2.8	2.95	69.5	68.95	2.55	2.85
G	0.333	0.325	73.95	73.65	2.55	51.35	6.55	6.75	91.2	80.35	2.8	2.95
H	0.245	0.229	2.8	2.9	35.5	33.5	72.6	75	76.5	71.95	48.3	45.5

Calibrator
Positive sample
Negative sample
Blank
Negative control
Positive control

$$\text{EU/ml of sample} = \frac{\text{EU/ml of Calibrator}}{\text{Absorbance of Calibrator}} \text{ X } \text{Absorbance of sample}$$

Figure 6.1: An illustrative example of a VZV specific IgG ELISA test

The picture is of the 96-well ELISA microplate that was used to detect VZV-specific IgG antibody. Each sample was replicated in two adjacent wells and the average of absorbance reading from both wells was calculated. The Table presents results of ELISA and the numbers indicate the concentration of VZV-specific IgG antibody as EU/ml calculated by the formula below the table. The conclusion for the VZV immunity is colour coded, see the table legend for each colour code.

6.5.2 Choosing the appropriate VZV- antigen for optimal T cell response

Based on the literature so far, most of the work on VZV T cell responses, particularly CD4+ T cell responses, were based on Limiting Dilution Responder Cell Frequency assay (RCF assay) (Asano *et al.*, 1993). Different antigens from different companies such as East Coast Biologics and ABI Company were used for this purpose (Klein *et al.*, 2006, Jones *et al.*, 2007). However, there is insufficient data concerning using these antigens in short term stimulation assays such as ICS assay. Therefore, the initial step was to investigate the competence of these antigens to generate an adequate T cell response that can be measured by assays developed in this project.

Briefly, 20ml of blood was taken from six consented adult volunteers. Four of them were VZV seropositive (having detectable levels of anti-VZV IgG antibody by ELISA) while the other two were seronegative. The immune responses were measured by detecting an increase in intracellular cytokines (IFNγ and TNFα) by flow cytometry. In addition to East Coast Biologics and ABI companies reagents another two different vaccine antigens from Varivax (Merck, NJ, USA) and Varilrix (GlaxoSmithKline, Rixensart, Belgium) were also tested in the current study. The efficiency of these antigens to serve as a good stimulator was determined by achieving a significant detectable responder T cell population. For details, see chapter 2, section 2.4.3.

The result of this experiment demonstrates that these different antigens can specifically trigger VZV-specific T cell responses, however, there was very mild non-significant difference in term of frequency of T cell response. This was true even when a mixture of all antigens was used to stimulate PBMC. Indeed, using one of the vaccines would be

more cost effective. Therefore, Varilrix vaccine (V.R) vaccine was used for rest of all experiments.

Since the full activation of T cells necessitate two signals (Signal 1 is through the TCR-MHC-peptide interaction and signal 2 is through the CD28:CD80/CD86 interaction), the requirement for T cell co-stimulation was also tested. Here anti-CD28 and anti-CD49d was added to each tube at the beginning of the incubation, the flow cytometric analysis showing that the T cell response against V.R vaccine could be enhanced by using of these co-stimulators. Therefore, in all subsequent assays these antibodies were integrated to detect the maximum possible response. A representative example of T cell response using V.R vaccine in adults is shown in Figure 6.2.

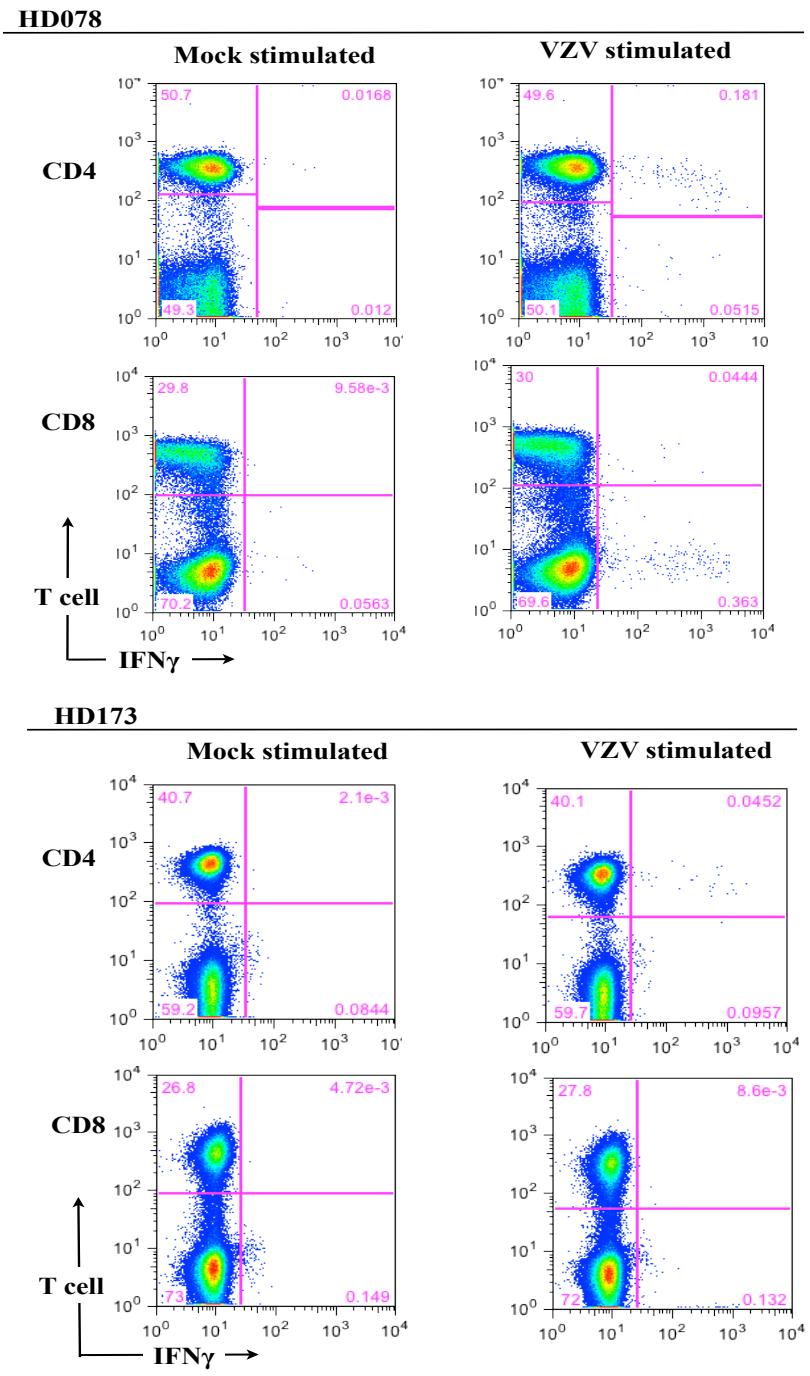


Figure 6.2: T cell responses in healthy donors to Varilrix vaccine

Flow cytometry plots showing T cell responses against Varilrix (VR) vaccine in PBMC of two VZV-seropositive healthy donors (HD078 & HD0173). Values shown in right upper quadrants indicate frequencies of the T cell subset producing cytokine (IFNγ) out of total lymphocytes. In both donors there was a stronger CD4 T cell response.

6.5.3 Differentiation between positive and negative T cell responses to VZV antigen

Similar to most laboratory tests reliant on visualization, one of the disadvantages of flow cytometry is the lack of a clear-cut discriminant value that distinguishes between positivity and negativity. This is particularly important when the difference is very small. To avoid possible subjective error in interpretation of results, besides the VZV-stimulated tube, a mock-stimulated tube was also used with each patient test. Furthermore, aiming to define clear parameters for positivity and negativity, the index of the increase in the frequency of cytokine producing cell was measured. This includes calculating any increase in the ratio of VZV-specific T cells in VZV-stimulated and Mock-stimulated tube using the formula below.

$$\textit{VZV T cell immunity} = \frac{A}{B} \; X\,100 \rightarrow$$ if the result ≥ the selected threshold indicates positive immunity

A= cytokine+ T cell % of total lymphocyte in ***VZV***-stimulated tube

B=cytokine+ T cell % of total lymphocyte in ***Mock***-stimulated tube

The interpretation of results was that any donor with VZV T cell immunity that exceeded a certain threshold was considered VZV-immunized. The threshold level was estimated using a receiver-operator characteristic (ROC) curve, see Figure 6.3. Basically, this test helps to visualize and understand the trade-off between the high sensitivity and high specificity. For instance, in this project, the response of CD4+ T cells to VZV was considered genuine if the frequency of responding cells in VZV-stimulated was higher than the response in the mock tube by at least 77%. This threshold was selected based on the best combination of sensitivity of 85% (95% CI=66.27% to 95.81%) and specificity

of 72.73 % (95% CI=66.27% to 95.81%) with likelihood ratio of 3.12. A similar approach was carried out to calculate the threshold for VZV-specific CD8+ T cells, which was an increment of 30% at sensitivity of 75% (95% CI=53.29% to 90.23%) and specificity of 60 % (95% CI=24.24% to 87.84%) with likelihood ratio of 1.88.

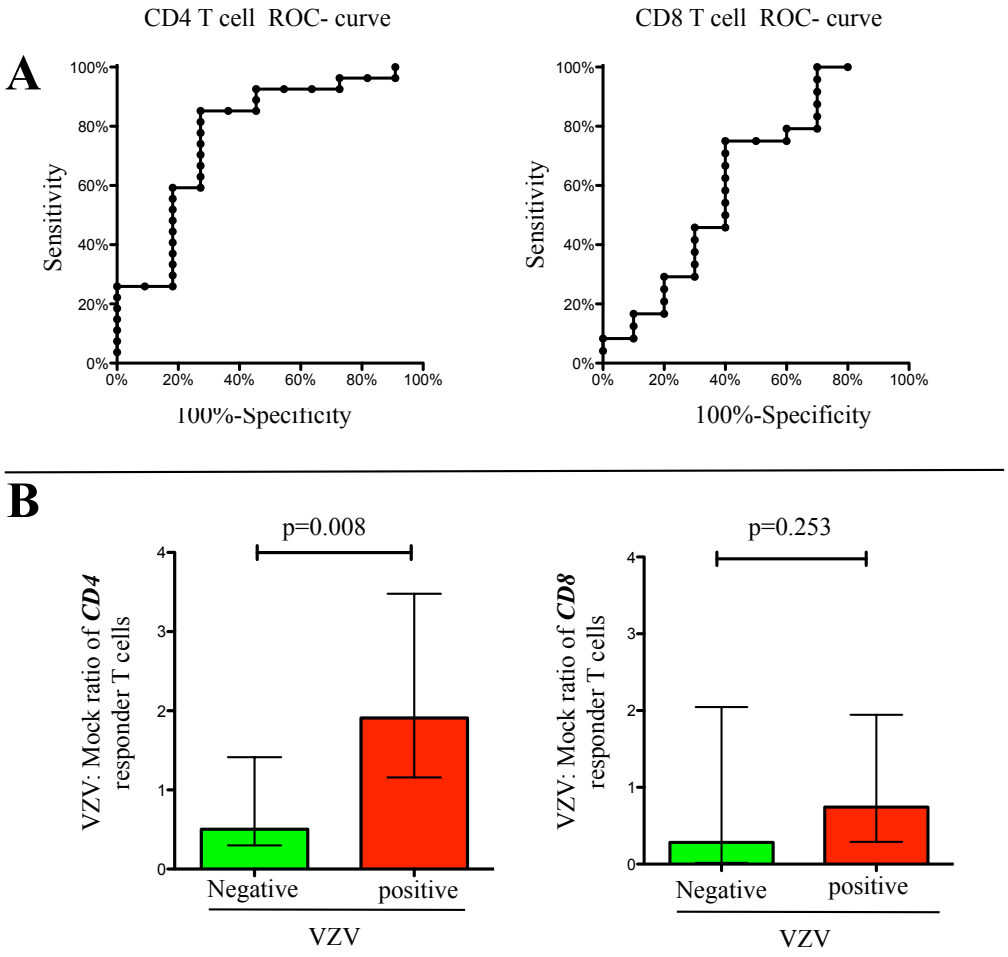


Figure 6.3: ROC-curve for VZV-specific T cell response in healthy donors.

Shows a representative example of how to the select the optimal threshold to differentiate between true responses and background based on sensitivity and specificity. ROC-curves (A) for both CD4 and CD8+ T cells were created by plotting the ratio of increase in VZV-specific T cell in VZV-seronegative and VZV-seropositive healthy donors. Graph (B) shows comparison between VZV-seropositive and seronegative group regarding the increment in the ratio of CD4 and CD8 VZV-specific T cell (right and left graph respectively). Columns and error bars indicate median with interquartile range respectively.

6.5.4 T cell response to VZV antigens in children with acute lymphocytic leukaemia

After optimizing the VZV-ICS assay in healthy controls, the next challenge was to investigate if the same assay would work in leukaemic children who were on chemotherapy including high doses of immunosuppressive drug such as prednisolone.

Since most patients were suffering from lymphopenia, besides the limited volume of blood sample that was permitted to be taken (8-12 ml/time-point), it was mandatory to downscale the PBMC number used per tube. Therefore, instead of the usual number of 0.5-1$\times 10^6$ PBMC per test only 0.1-0.3 $\times 10^6$ PBMC per tube was used. Indeed, this necessitated downscaling of all other reagents including the quantity of VZV antigen, co-stimulator, and Brefeldin-A to optimal concentrations. Besides the low number of PBMC there was another issue, which is the effect of chemotherapy on the cell viability. Therefore, the goal was to process the samples as quickly as possible after they had been collected.

Examples of T cell responses to Mock, VZV antigen, and OKT3 in leukaemic children are shown in Figure 6.4. CD4+ T cells and to less extent CD8+ T cells showed an increase in production of IFNγ and TNFα after stimulation with V.R vaccine and after stimulation with OKT3 as positive control compared to the mock tube (negative control).

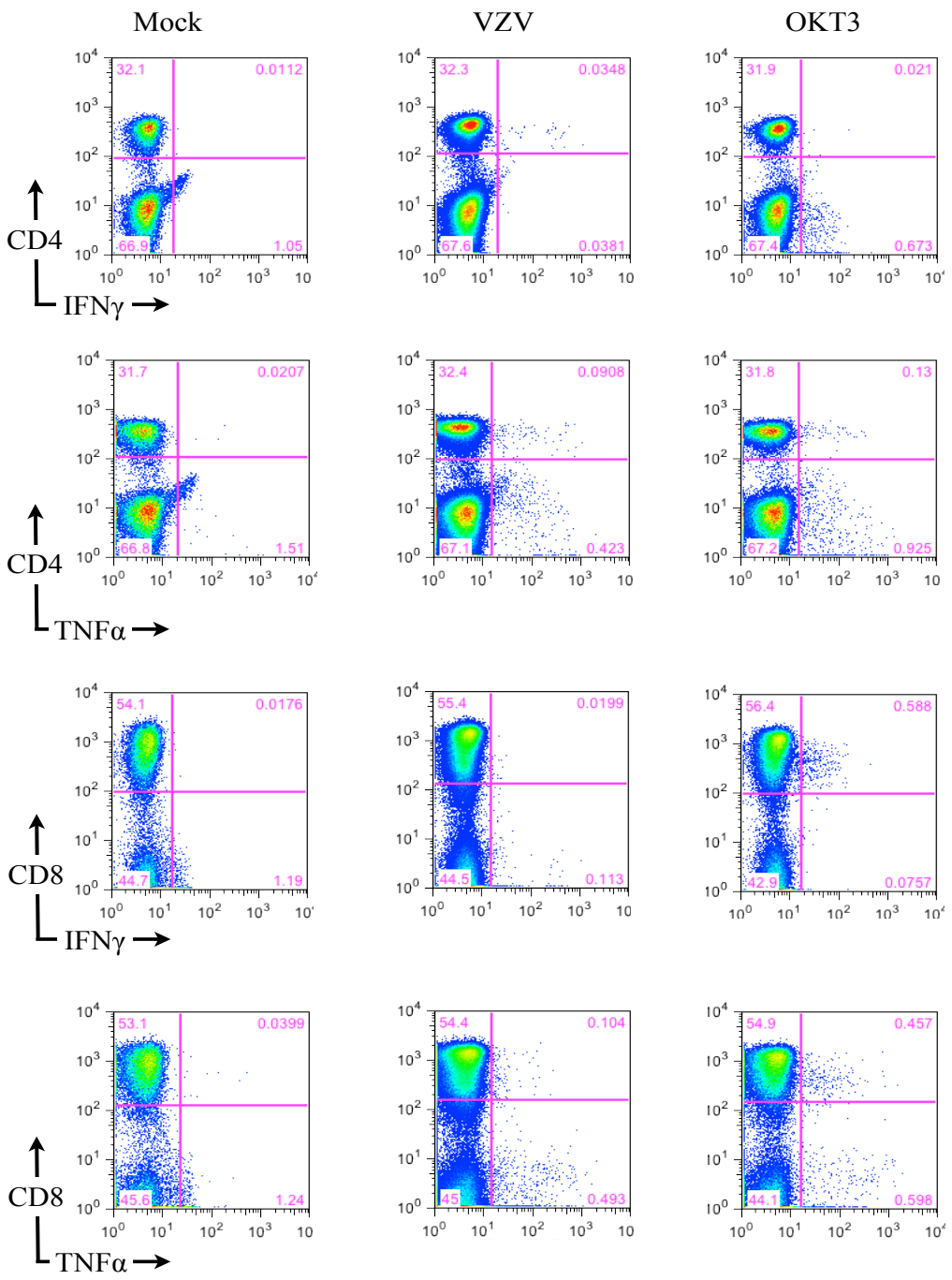


Figure 6.4: Illustrative example of T cell response to different antigens in ALL children.

This illustrates the CD4+ T cell response (upper two rows of plots) and the CD8+ T cell response (lower two rows of plots) by measuring levels of intracellular cytokine (IFNγ and TNFα) in response to different stimuli including; Mock, VZV antigen, and OKT3. Numbers inside the quadrants indicate the frequency of different lymphocyte subsets as % of total lymphocytes.

6.5.5 Both VZV-specific CD4+ and CD8+ T cell responses can be detected in healthy individuals by CFSE proliferation assay

Taking into consideration that T cell response to antigen is not only restricted to effector function but also includes the ability to proliferate or to induce proliferation in other cell subsets, the aim here was to investigate the ability of VZV-specific CD4+ and CD8+ T cells to proliferate in response to VZV antigen (V.R vaccine) utilizing a CFSE proliferation assay. PBMC from healthy control donors with good ICS assay response were used for standardizing this assay. Briefly, after isolation of PBMC in the standard way the PBMC: media concentration was corrected to 1×10^6 PBMC/ml then CFSE stain was added at appropriate concentration. This was followed by incubation with different antigens including; PBS (as Mock), V.R vaccine, CMV lysate, influenza vaccine, and OKT3 (as positive control). For more details about this technique, see section 2.4.4.

A representative example is shown in Figure 6.5. It demonstrates that VZV specific CD4+ T cells and to a lesser degree CD8+ T cells can proliferate in response to VZV antigen. The level of proliferation was similar to that induced by influenza vaccine. However compared to the CMV and OKT3 response it was much weaker.

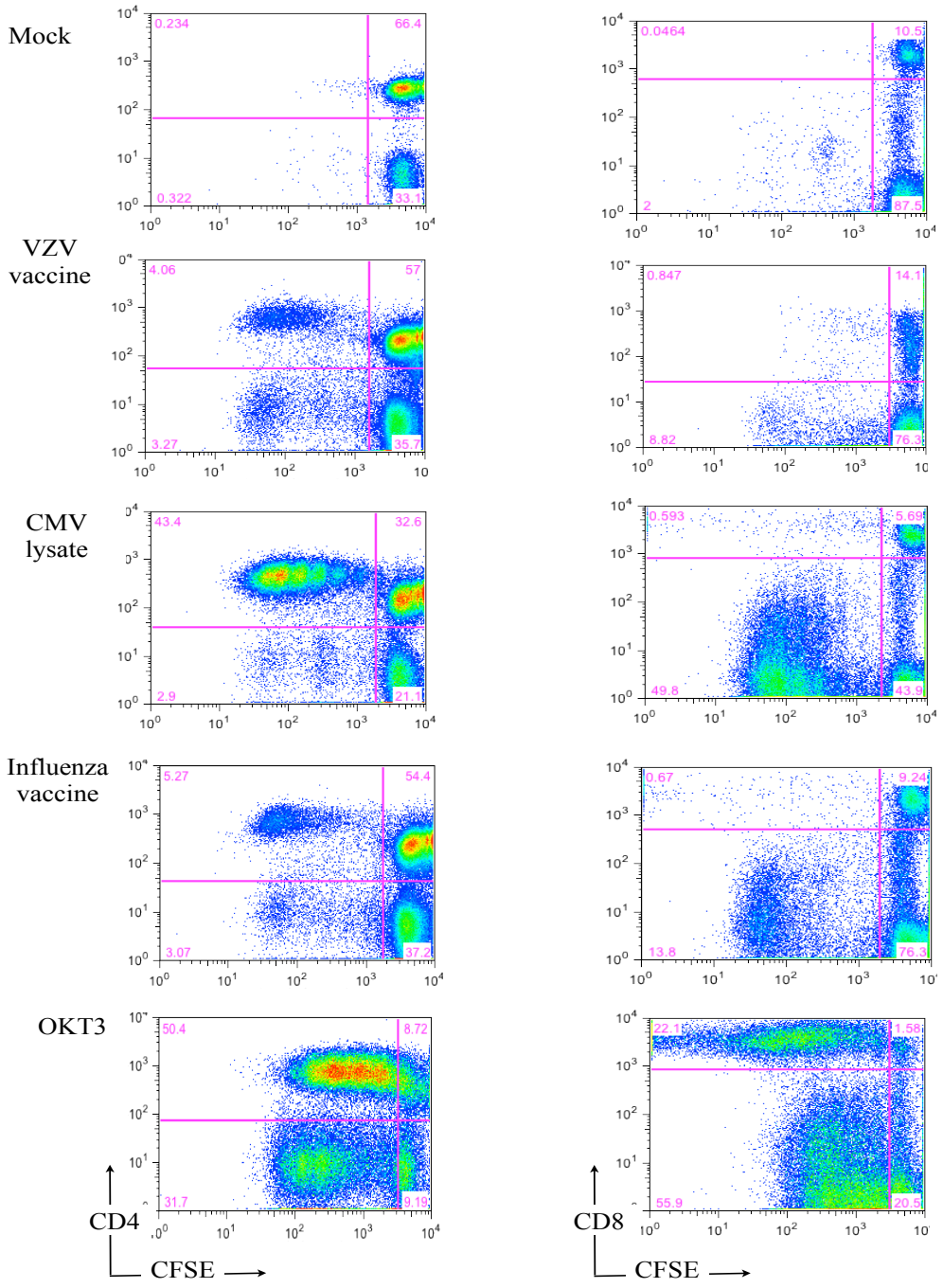


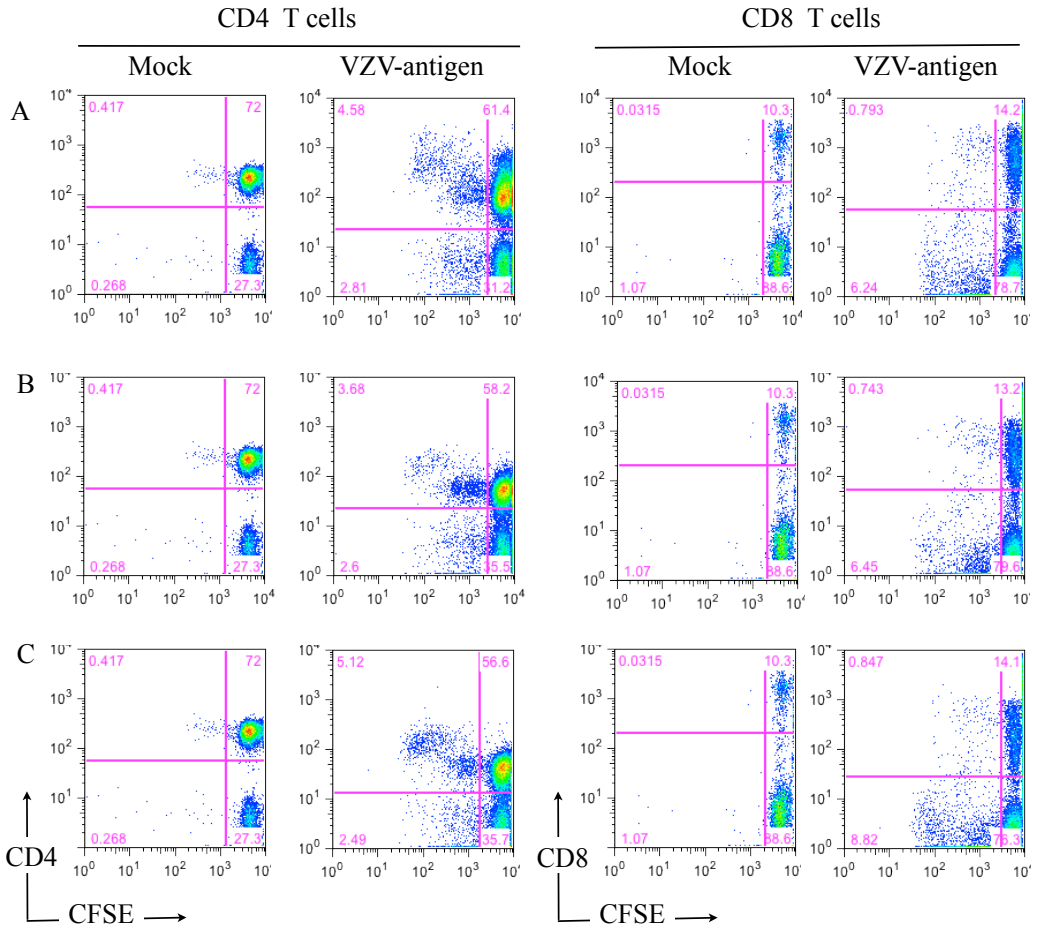
Figure 6.5 A representative example of CD4+ and CD8+ T cell proliferation response in a healthy donor.

PBMC dot plots show the proliferation of CD4+ T cells (left column of plots) and CD8+ T cell (right column of plots) in the same healthy donor. Different antigens were used including; mock, V.R vaccine, CMV lysate, influenza and OKT3. The frequency of each cell subset is shown inside of each dot plot quadrant.

6.5.6 Implementation of proliferation assay in leukaemic children

Since as mentioned above regarding viability and the low number of lymphocytes in patients samples, it was predicted that establishment of the proliferation assay in leukaemic children would not be an easy task. Therefore, the first step toward achieving this aim was optimising this assay to work with few numbers of cells in healthy control adults. The goal was to start with as low a number of PBMC as possible. Hence, downscaling of initial PBMC count from 1-2 x 10^6/ ml and V.R vaccine concentration of 10µl per culture well (2 ml final volume) into 0.1-0.2x10^6/ ml and V.R vaccine concentration into 1µl per culture well (0.5ml final volume) was carried out. Results of this experiment are demonstrated in Figure 6.6. Here, T cell proliferation was achievable even with the use of 0.2x10^6/ ml PBMC and 1µl V.R vaccine per culture well (0.5ml final volume).

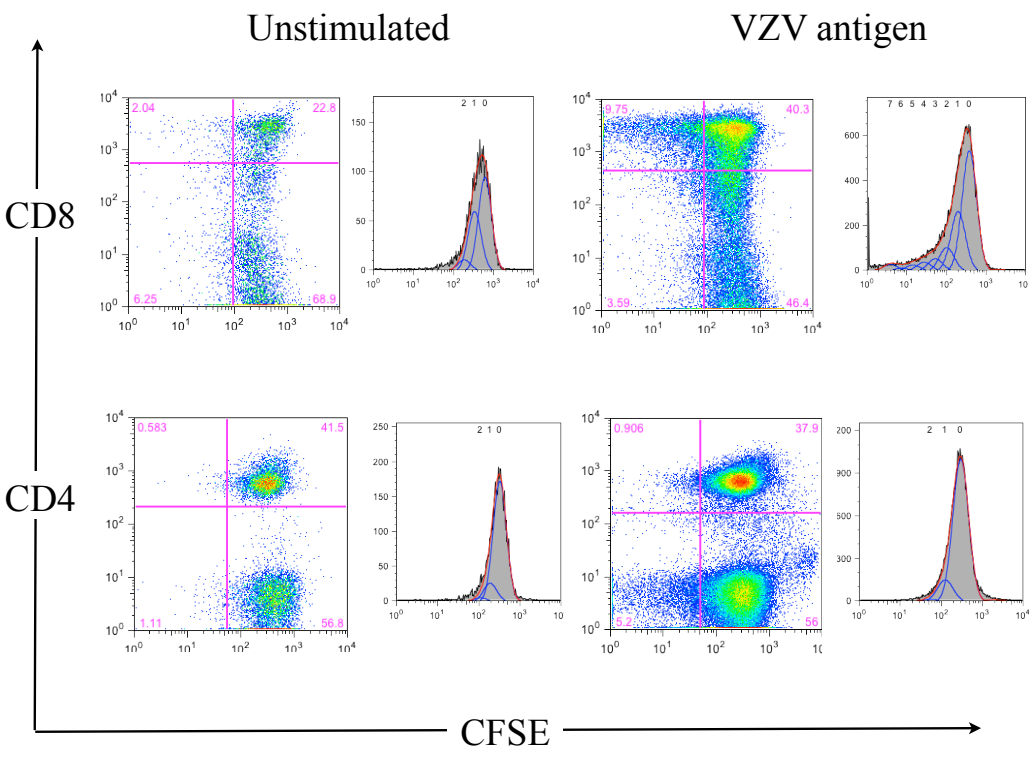
Despite the achievement in downscaling of the proliferation assay to be applicable to low lymphocyte numbers, applying this assay in leukaemic patients was successful only in a few instances. The high nonspecific background in mock tubes led to difficulty in interpretation of results. One example of the few relatively successful experiments is shown in Figure 6.7.



Panel of plots	Initial PBMC count per ml	VZV antigen (V.R vaccine) concentration µl/ml	Final volume /well
A	1x106	10	1
B	0.5x106	5	0.5
C	0.2	1	0.5

Figure 6.6: Downscaling of T cell proliferation assay

Panel of plots are the proliferation assay of CD4+ and CD8+ T cell responses to VZV vaccine in healthy donors. Initial PBMC count, V.R vaccine concentration and the final volume per each culture well used for each panel are shown in the table below.



Donor/ALL36	CD4 T cells		CD8 T cells	
Antigen	Mock	VZV	Mock	VZV
Proliferation Index	1	1.08	1	1.32
%Divided	8.75	7.68	14.4	24
Replication Index	1.09	1.09	1.14	1.52
Significant difference in T cell response	NO		YES	

Figure 6.7: An example of a proliferation assay in leukaemic children.

Plots and accompanying histograms are an illustration of mock (left column) and VZV-specific (right column) CD8 and CD4 T cell proliferation assays (upper and lower panel of plots respectively) in a leukaemic patient (ALL036). The Table is a summary of some statistical values generated from the histograms. **Proliferation Index**: indicates the total number of divisions divided by the number of cells that went into division; **% Divided** denotes The percentage of cells from the original sample, which have divided, and **Replication Index** means the average number of cell divisions that a cell in the original population has undergone and is equal to (Proliferation Index) X (% Divided).

6.5.7 History of chickenpox might infer the VZV T cell immune status

In normal situations a history of previous VZV infection is usually associated with long-lived B and T cell protection; whether this association is also true in context of leukaemia is not well known. To address that, the association between the histories of VZV in leukaemic children was compared to both the ELISA results (as indicator of B cell function) and ICS assay results (as indicator of T cell function). See Tables 6-2 and 6-3. Fisher's exact test confirms the presence of an association between history and serology of VZV $p<0.0001$. However, the association between history of VZV infection and T cell immunity was less significant.

Table 6-2: Association between VZV infection history and B cell immunity

Association between previous history of VZV and VZV-ELISA results		VZV-ELISA		
		VZV-positive	VZV-Negative	Equivocal
History of VZV infection in 70 patients	Positive= 41	30	7	4
	Negative= 29	3	21	5
Total		33	28	9
Comments		Fisher's exact test P<0.0001 Sensitivity= 90% Specificity =75%		

Table 6-3: Association between VZV infection history and T cell immunity

Association between previous history of VZV and VZV-specific T cell response		VZV-specific T cell response		
		VZV-positive	VZV-Negative	Equivocal
History of VZV infection in 70 patients	Positive= 41	24	17	0
	Negative= 29	7	20	2
Total		31	37	2
Comments		Fisher's exact test P=0.0125 Sensitivity= 77% Specificity =54%		

6.5.8 Change in the CD4:CD8 T cell ratio in leukaemic children.

In the current study, basic lymphocyte counting showed that most leukaemic children had lymphopenia (median with upper and lower interquartile = 0.6 (0.4-0.9) $x10^6$/ml of blood. This level of lymphocyte numbers is substantially low compared to both the normal range in healthy children (age group of 1-18yr) of 3.1 (2.3-3.15) $x10^6$/ml and to healthy adult controls (current study) of 2.0 (1.6-2.5) $x10^6$.

Considering that, it was not clear whether this reduction in the total lymphocyte count was affecting the whole T cell population, whether it was associated with proportionate reduction in different T cell subsets or a decrease in only one T cell subset more than the other. To investigate this, both frequency and absolute numbers of CD4+ and CD8+ T cell were measured by flow cytometry and then the CD4:CD8 T cell ratio was calculated. Figure 6.8 illustrates steps to identify CD4+ and CD8+ T cells in healthy adults and leukaemic patients. Meanwhile Figure 6.9 displays the percentage and ratio of these subsets in patient groups comparing both healthy adult controls and healthy children (reference range obtained from previous study (Schatorje *et al.*, 2012)).

Interestingly there was a significant difference ($P < 0.0001$) in the CD4:CD8 T cell ratio in leukaemic children compared to healthy control adults of current study, which also seems to be present even when compared to healthy children Figure 6.9. However, it is not clear if this difference is attributable to the expansion in CD8+ T cells or reduction of the CD4+ T cell population.

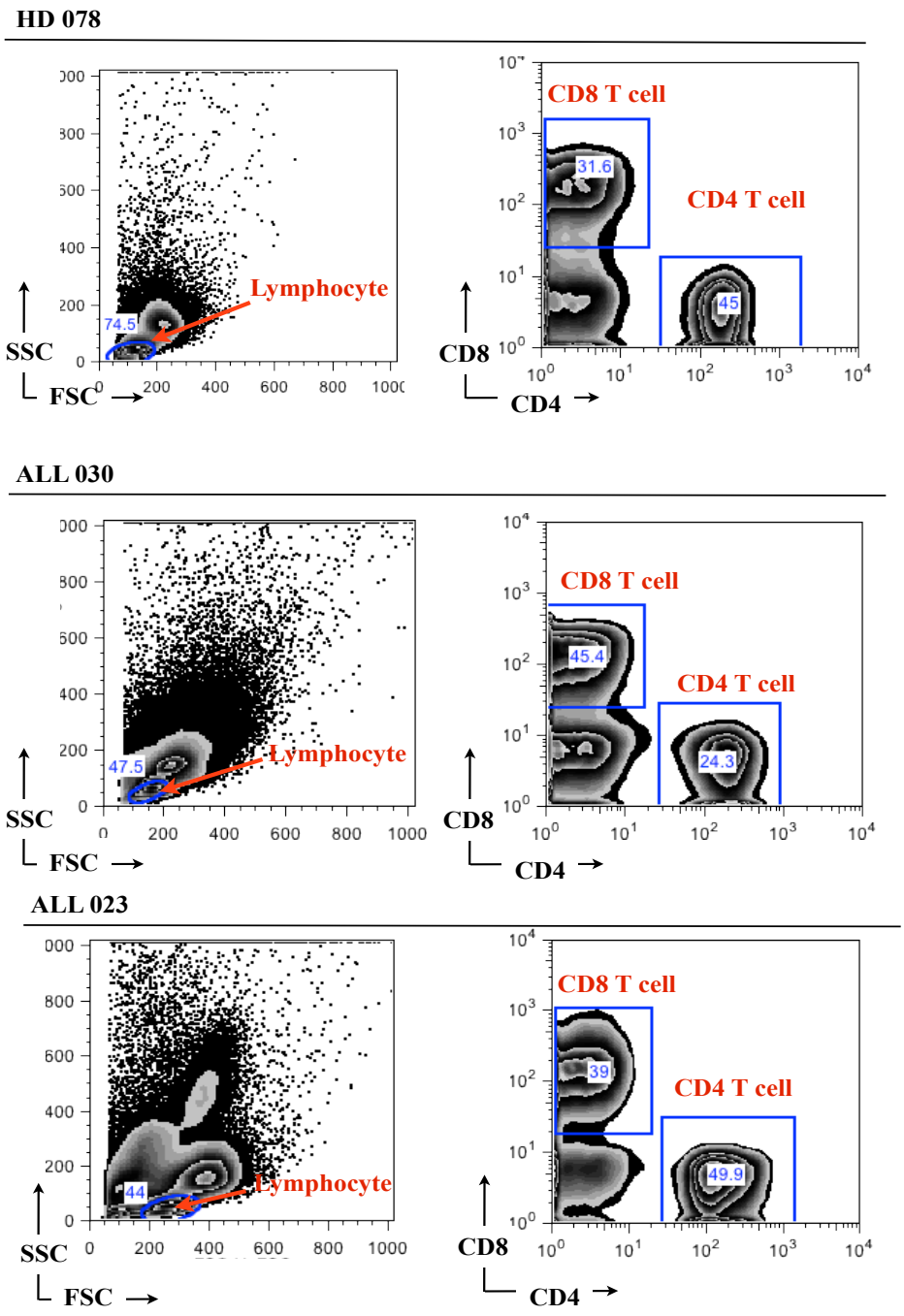
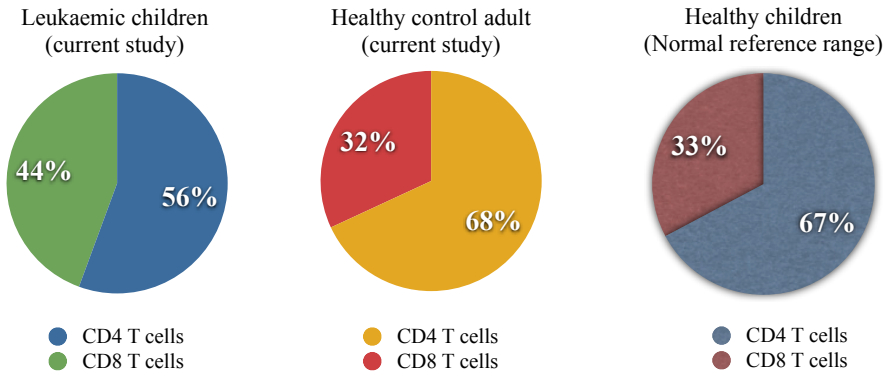


Figure 6.8: Identifying frequencies of CD4 and CD8+ T cells

This demonstrates an illustrative example of CD4 T cell and CD8 T cell frequency in a healthy control (HD 078) and leukemic children (ALL 23 and ALL30). Firstly, an original gate was drawn around the lymphocyte population on the FSC and SSC plot followed by sub-gating on CD4+ and CD8+ populations to identify CD4 T cell and CD8 T cell frequencies in the original gate.



	Leukaemic children	Healthy adults (control current study)	Normal reference range in healthy children	Difference between leukaemic children and control healthy adult
lymphocyte count $X10^6$ per ml of blood	0.6 (0.4-0.94)	2.0 (1.6-2.5)	3.150 (2.325-3.150)	P value < 0.0001
CD4 T cells % of total lymphocyte	39.19 (27.92-54.41)	51.45 (43.48-57.10)	41 (38-46)	P value =0.0012
CD8 T cells % of total lymphocyte	30.32 (19.01 27.90)	23.65 (19.68-38.58)	20 (17-25)	P value= 0.0437
CD4:CD8 ratio	1.401 (0.82-2.47)	2.12 (1.70-3.01)	2.05 (1.57-2.87)	P value = 0.0017

Figure 6.9: Comparison between distributions of total CD4+ and CD8+ T cell in healthy controls and leukaemic children.

Pie charts demonstrate the percentage of CD4+ and CD8+ T cells in peripheral blood of leukaemic children, healthy adult controls and healthy children. The Table summarises the differences in the absolute lymphocyte counts, as well as differences in the frequencies and CD4:CD8 T cell ratios. Values denote the median with upper and lower quartiles. P values were calculated using the Mann-Whitney test with 95% confidence intervals.

6.5.9 Significant reduction in the absolute numbers of CD4+ and CD8+ T cells in leukaemic children.

The change of CD4:CD8 T cell ratio in association with lymphopenia might suggest that there was a disproportionate reduction in CD4+ and CD8+ T cells. However, it is not clear, if this difference could be attributed to expansion in CD8+ T cells after reduction of the CD4+ T cell population or as a consequence of reduction in both populations that was more profound in CD4+ T cells. To answer this a comparison between absolute numbers of CD4+ and CD8+ T cells in leukaemic children and healthy control was carried out (Figure 6.10).

Although, apparently there was a high frequency of CD8+ T cells in patient samples, the comparison of absolute numbers showed that both CD4+ and CD8+ T cells were significantly less in leukaemic patients. This might support the proposal that there was unbalanced CD4+ and CD8+ T cell populations reduction that probably affected CD4+ T cells more than CD8+ T cell populations.

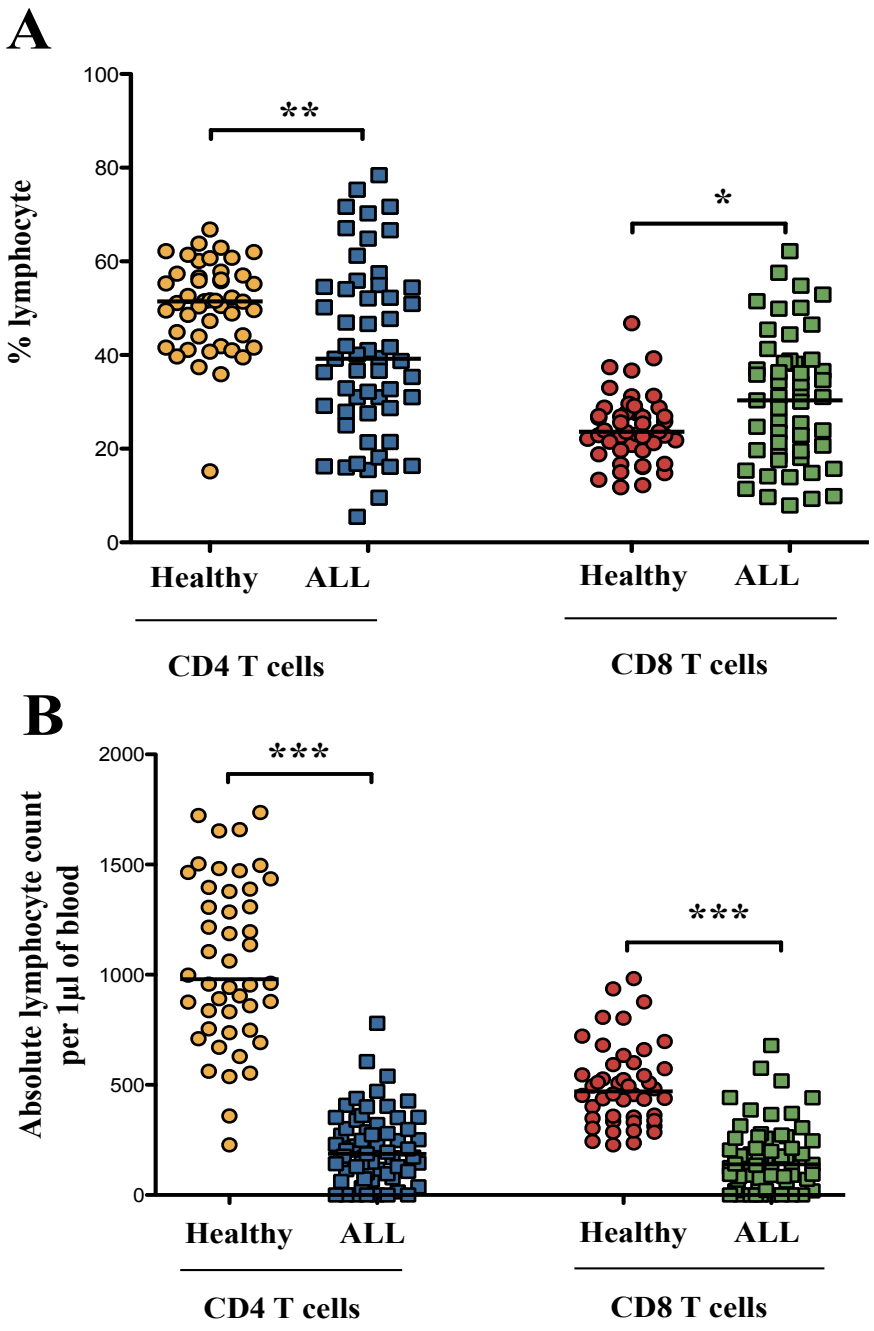


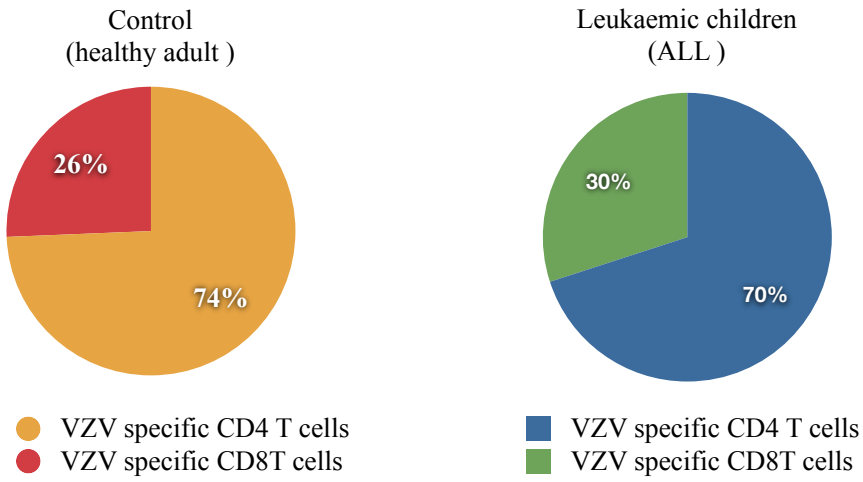
Figure 6.10: Comparing frequency and absolute CD4+ and CD8+ T cell numbers in leukaemic children and healthy adult controls.

The graph displays differences in the frequencies (**A**) and absolute counts (**B**) of T cell populations in healthy adult controls (CD4 and CD8; yellow and red spots respectively) and leukaemic children (CD4 and CD8; blue and green squares respectively). (ns= not significant, *= $p<0.05$, **= $p<0.001$, and ***= $p<0.0001$).

6.5.10 VZV-specific T cell distribution is comparable in healthy controls and leukaemic children.

Having observed the disproportionate shrinking of the total T cell population this led to another question about whether VZV-specific T cells follow the same pattern of distribution as the total T cells or not. To answer this question the frequencies of VZV-specific CD4+ and CD8+ T cells were identified and the ratio between them was calculated in VZV seropositive patients and control groups. Here flow cytometry and ICS assays were utilised to measure the response of T cells to VZV antigen by detecting any increase in intracellular cytokine. Note, here both anti-IFNγ and anti-TNFα monoclonal antibodies were conjugated to the same flurochrome, hence, any increase in either of these cytokines will indicate VZV specificity.

Interestingly, opposite to expectation, the frequency of VZV-specific CD4+ and CD8+ T cell in leukaemic children (median; 0.42 and 0.16 respectively) was very similar to that median of frequency of VZV-specific CD4+ and CD8+ T cell seen in healthy adult controls (0.53 and 0.22 respectively). Furthermore, there was no significant difference in the CD4:CD8 ratio between both groups ($p>0.05$). See Pie charts and integrated table in Figure 6.11.



	Leukaemic children	Healthy adults	Difference between leukaemic children and control healthy adult
VZV-specific CD4 T cells % of total lymphocyte	0.42 (0.14-1.05)	0.53 (0.20-1.23)	P value > 0.05
VZV-specific CD8 T cells % of total lymphocyte	0.16 (0.06-0.45)	0.22 (0.07-0.63)	P value > 0.05
CD4:CD8 ratio	2.2 (0.82-4.35)	1.74 (0.81-4.51)	P value > 0.05

Figure 6.11: Comparison between healthy controls and leukaemic children VZV-specific T cell distribution.

Pie charts show the percentage of VZV-specific CD4 and CD8+ T cells out of total lymphocytes in both leukaemic and control groups. The Table summarises the comparison between the two groups. Values denote the median with upper and lower quartiles. P values were calculated using the Mann-Whitney test with 95% confidence intervals.

6.5.11 VZV-specific T cell response in healthy and patient groups has a different cytokine profile.

Although there was a similarity in the frequency of VZV-specific T cells between patient and control groups, the subcategorizing of these cells according to type of cytokine profile against VZV antigen revealed various differences. This time each cytokine (IFNγ and TNFα) was stained with a different flurochrome. Accordingly, the VZV-specific T cell population was sub-classified into four subsets that include: IFNγ+/CD4, TNFα+/CD4, IFNγ+/CD8, and TNFα+/CD8 VZV-specific T cells.

The VZV T cell response in healthy populations is characterized by dominance of IFNγ+/CD4+ T cells, which comprise approximately two thirds of responding T cells see Figure 6.12(A). In contrast, the chief population in the patient group was TNFα+CD4+ T cells (47% of responding T cells). Meanwhile, the VZV-specific CD8+ T cell population in both the control and the patient groups consisted of slightly larger TNFα+CD8+ T cell populations.

Since the difference in the frequency of VZV-specific T cells does not necessarily indicate a difference in the absolute numbers of VZV-specific cells, a comparison in the absolute count of these subsets per microlitre of blood between the patient group and healthy adult control group was carried out (Figure 6.12,B). The only significant difference was between IFNγ+/CD4 T cell populations, which were remarkably low in the patient group (median, interquartile; 0.2, 0.1-0.6) per 1μ of blood compared to the healthy control group (median, interquartile; 0.79, 0.3-1.4).

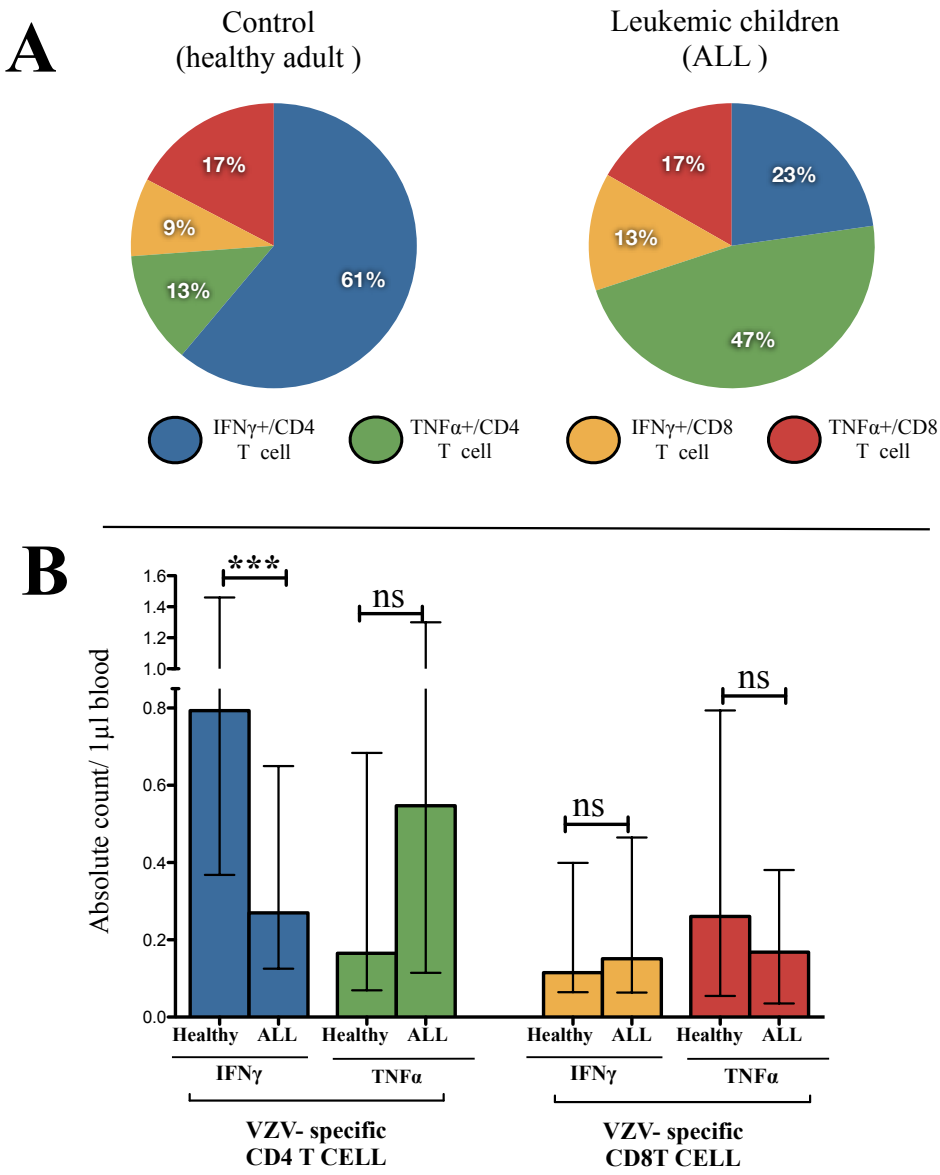


Figure 6.12: Difference in cytokine profile of VZV specific CD4 and CD8+ T cells in healthy adult controls and leukaemic children.

Pie charts (A) demonstrate the percentage of contribution of each subset of the VZV specific T cell response in healthy control and leukaemic children (ALL). Each colour denotes a different subset of cells: blue =IFNγ+/CD4, green =TNFα+/CD4, yellow =IFNγ+/CD8, and red =TNFα+/CD8 VZV specific cells. The graph (B) shows the differences in the absolute counts of these subsets per 1µl of blood in healthy controls and patients (ALL). The top of each column indicates the median with error bars showing the interquartile range; ns= not significant, *= $p<0.05$, **= $p<0.001$, and ***= $p<0.0001$).

6.5.12 Sub-classification of leukaemic children according to VZV clinical infection.

Having observed the difference in the cytokine profile between healthy control and patient groups, and taking into consideration that not all leukaemic children experienced VZV infection, it was reasonable to ask if there were differences in the quality of T cell response to VZV within the patient group. In other words, is there any specific T cell profile that might play a role in protection against or susceptibility to a new VZV infection or reactivation? Note, the quality of T cell response here is referred to the frequency of the responding cells as well as their cytokine profile.

To answer this question, firstly the patient groups were classified according to the history of pre-leukemic VZV infection into two groups: patients with positive history (n=41 patients) and those with negative history (n=29). Postulating that the positive history group are VZV immunized (see section 6.5.7), they were subdivided into three subgroups; this included (Group A) which includes leukaemic children who had no clinical VZV infection during the chemotherapy and their ELISA were negative for VZV antibodies, they are only 5 patients. Group B involved leukaemic children who had no clinical VZV infection during the chemotherapy but their ELISA were positive for VZV antibody (23 patients). Finally, Group C comprised children who experienced clinical VZV infection regardless of the serology (13 patients).

Since the aim was to compare the T cell profile in relatively VZV protected leukaemic children to that of susceptible leukaemic children, each group was selected according certain criteria. For instance, in the 'no clinical VZV infection' groups (group A and B), only those patients who achieved advanced chemotherapy maintenance cycles, such as

cycle 8, 10, or EOT (End Of Therapy) with no VZV infection were included. Meanwhile, group C includes any patient who had chickenpox or herpes zoster regardless of the stage of the treatment. However, for group C only the sample preceding an episode of clinical VZV infection was considered. For instance, if a patient developed VZV between maintenance cycles 2 and 4, the T cell profile during cycle 2 would be considered for this comparison. Meanwhile, the sample taken during acute infection and the cycle 4 sample (post infection) would not be included for this purpose.

The basic initial comparison was to compare the absolute counts of lymphocytes, total CD4 T cells, and total CD8 T cells. In addition, the difference in CD4:CD8 T cell ratio between groups was determined. These are summarised in Table 6-4.

Table 6-4: Classification of leukaemic children with positive history of VZV infection into three subgroups

Leukaemic children	**Group A**	**Group B**	**Group C**	Statistical Differences between ***Group C*** and	
				Group A	***Group B***
History of pre-leukaemia VZV infection	Yes	Yes	No / Yes		
clinical chickenpox or herpes zoster during chemotherapy	No	No	Yes		
ELISA for VZV antibody	negative	positive	negative or positive		
Absolute total lymphocyte count per µl of blood	590 (500-970)	450 (300-690)	900 (490-1740)	$p>0.05$	$p<0.05$
Absolute CD4 T cell count per µl of blood	280 (205-351)	141.6 (69.8-264.3)	205.8 (151-633)	$p>0.05$	$p>0.05$
Absolute CD8 T cell count per µl of blood	200 (121-319)	101.6 (48.82-188)	200 (93-489)	$p>0.05$	$p>0.05$
CD4:CD8 ratio	1.2 (0.71-0.32)	1.59 (1.04-2.04)	1.29 (0.90-1.8)	$p>0.05$	$p>0.05$

6.5.13 Decrease in the frequency of VZV-specific CD4+ T cells in VZV clinically diagnosed group (group C)

Considering that no statistical difference, neither in frequencies nor in the ratio of total CD4+ and CD8+ T cells between patient subgroups was observed, the next step was to compare the VZV-specific T cell profiles of these patient subgroups. This involved investigating the difference in CD4:CD8 T cell ratio of the VZV-specific population, as well as the difference in cytokine profiles between these subgroups.

The most interesting finding was the inverted ratio of VZV-specific CD4:CD8 T cell in leukaemic children with clinical VZV infection (Group C). For instance, this ratio was 0.72 in group C compared to 2.12 and 1.2 in group A and B respectively, see Figure 6.13 (I). Furthermore, in contrast to the dominance of TNFα producing CD4+ T cells in group A and B, approximately one third of VZV-specific T cells in group C was attributed to CD8+ T cells that mainly produce IFNγ, see Figure 6.13 (II).

In addition to the difference in VZV-specific T cell profile there were also differences in the absolute numbers. For instance, comparison between group C on one hand and groups A and B on the other hand showed a remarkably low absolute count of VZV-specific IFNγ+ CD4+ T cells in group C (median, interquartile; 0.12, 0.06-0.26/ µl blood). This difference was only statistically significant when group C was compared to group A ($p<0.05$), see Figure 6.13(III).

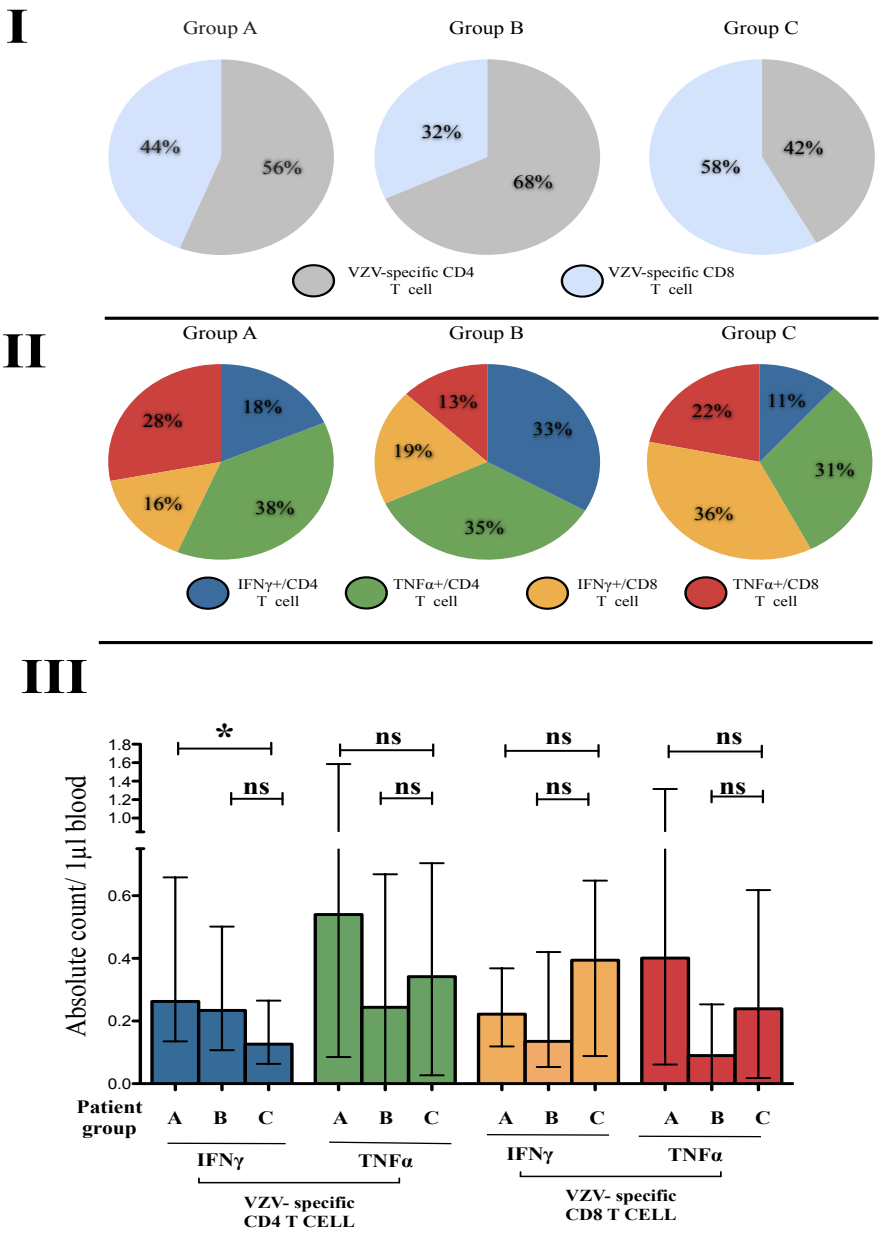


Figure 6.13: VZV-specific T cell profile in subgroups of leukaemic children

This compares T cell response to VZV antigen in leukaemic children subgroups, where Group A and B denote patient with no clinical infection and VZV-ELISA was negative and positive respectively. Group C includes patients with clinical infection. Pie charts (I) show the frequency of VZV-specific CD4 and CD8+ T cells in these groups. Meanwhile, Pie charts (II) demonstrate the cytokine profile of each group. The graph (III) compares the absolute count of each T cell subset in these subgroups. Note, ns= not significant, *= $p<0.05$, **= $p<0.001$, and ***= $p<0.0001$.

6.5.14 Monitoring of the loss of VZV-specific B and T cell immunity in leukaemic children.

Since the main goal of the current study was to identify a reliable immune risk profile that can predict leukaemic children who might be at higher risk of VZV new infection and/or reactivation, therefore, loss of either humoral and /or cellular immunity preceding VZV infection was proposed. Thus, the dynamic change in the VZV-specific T cell response and VZV-specific IgG antibody titre were measured. Table 6-5 below summarizes changes in the VZV immune status according to both assays.

Table 6-5: Summary of VZV-immune status of leukaemic children.

VZV-Immunity	Acquired (Negative ➡ Positive)	Lost (Positive ➡ Negative)	No change		Inconclusive
			(Negative ➡ Negative)	(Positive ➡ Positive)	
T cell (ICS assay)	11	8	22	15	18
B cell (ELISA)	6	4	15	25	24
Both T and B cell	2	2	10	8	52

*Note, 'inconclusive' includes both equivocal results and results if only a single time point per patient was tested.

Throughout the follow up of the cohort population, thirteen patients had clinical VZV infection and only four of them suffered from clinically diagnosed herpes zoster. Furthermore, approximately half of them have no history of VZV infection prior to leukaemia, which might suggests a primary infection.

6.5.15 Inversion in the VZV specific CD4:CD8 ratio might be a warning sign preceding appearance of clinical VZV.

Here, dynamic changes in VZV-specific B and T cell immunity in three different patients who experienced clinical VZV infection during chemotherapy course are presented. The first patient (ALL09) is an example of VZV primary infection (chickenpox), meanwhile, ALL23 and ALL24 are representative examples of reactivation (herpes zoster). See Figure 6.14.

Starting with ALL09, who is a male of 3yrs old with no history of VZV and was seronegative, he developed chickenpox two weeks after cycle 10. It was speculated that the actual viral infection and changes in the immune system might have started just around cycle 10. Herein, despite the lymphopenia ($0.63X10^6$/ml of blood) the level of VZV-specific T cells became detectable with a remarkable increase in the frequency of IFNγ+/CD8+ T cells. Nevertheless, there was no similar increase in the count of IFNγ+/CD4+ T cells, which possibly led to inversion of the CD4:CD8 T cell ratio. As predicted the VZV-specific IgG antibody titre was low at cycle 10 and surged up just around the time of appearance of clinical VZV.

The scenario in patient ALL23 was slightly different, as he initially had a primary infection and then reactivation while he was under chemotherapy. The first infection was between cycle 6 and 8, seemingly a primary infection as the patient had no prior history of chickenpox and his VZV-ELISA was negative. The second infection occurred near the end of therapy and clinically it affected multi-dermatomes, which perhaps supports the possibility of reactivation of latent virus. Since the period between cycle 6 and primary infection was more than 4 months then the immune profile at cycle 6 might not

be correlated to the primary infection. In contrast, only 4 weeks were separating cycle 10 from the second infection, therefore, the changes in immune profile might be related to VZV reactivation.

In cycle 8, which was three weeks after the primary infection there was a remarkable increase in the count of TNFα+/CD4 and TNFα+/CD8+ T cells. In contrast the counts of IFNγ+/CD4 and IFNγ+/CD8+ T cells during cycle 8 were very similar to their counts during the primary infection. However, for both IFNγ and TNFα producing T cells the CD4:CD8 ratio of VZV specific T cell was maintained above 2.0.

In cycle 10, with the exception of TNFα+/CD4+ T cells that dropped from their previous spike, there were increases in the counts of all other VZV-specific T cells. However, there was a remarkable change in the CD4:CD8 ratio of these cells.

Interestingly, in contrast to the T cell response, the anti-VZV IgG antibody titre steadily increased throughout the course of therapy and even before the appearance of the second infection.

Lastly, patient ALL24, who had a history of chickenpox before he developed leukaemia, was also VZV seropositive at beginning of the study. Between cycle 2 and 4 he developed herpes zoster. Similar to ALL23 there was inversion in the ratio of both TNFα+/CD4: TNFα+/CD8 and IFNγ+/CD4: IFNγ+/CD8 VZV-specific T cells in the sample preceding the appearance of infection.

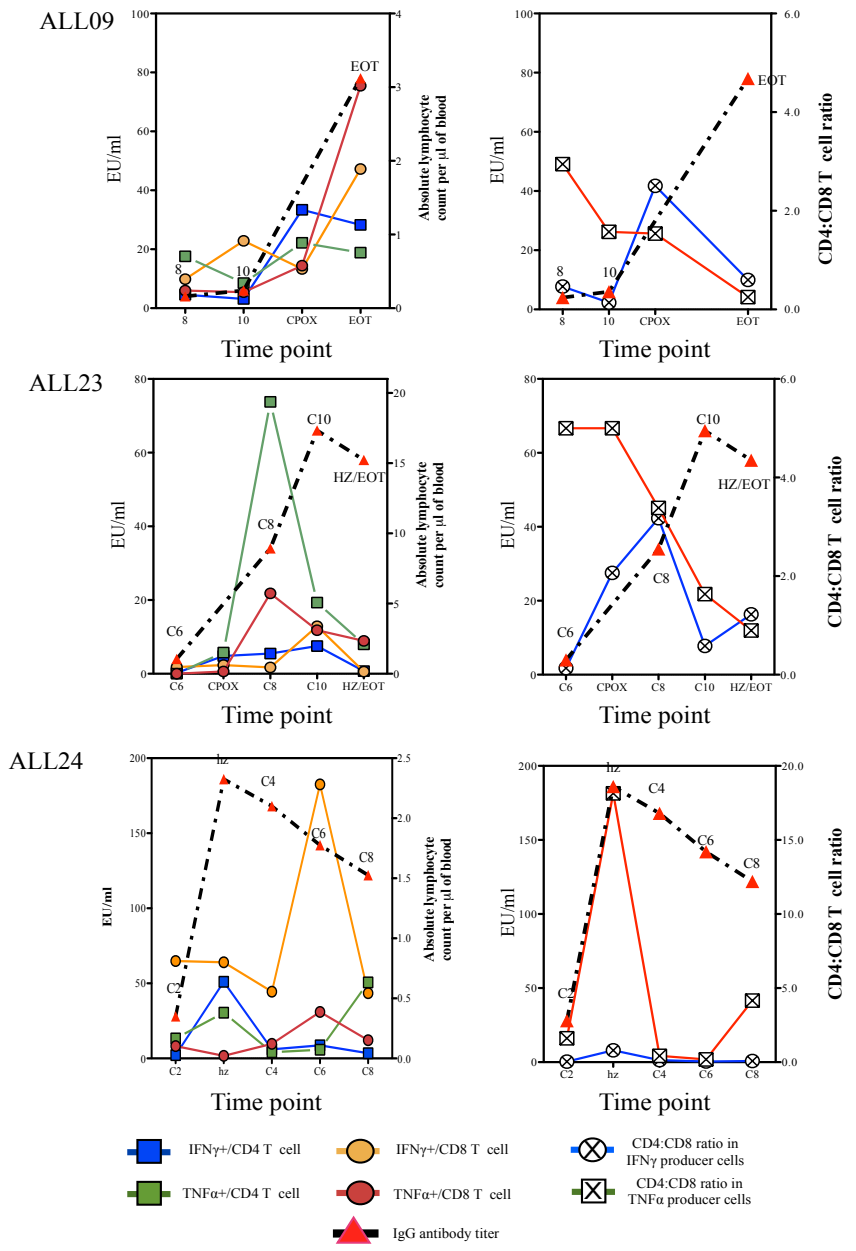


Figure 6.14: Changes in the level of VZV-specific T cell and anti VZV antibody titre in leukaemic children.

This demonstrates the dynamic variations in different subsets of VZV-specific T cell compared to changes in anti VZV IgG antibody titre during different cycles of chemotherapy (X axis) in three leukaemic patients. Left column of graphs shows the change in the absolute count of these cells (y axis). Meanwhile, the right column of graphs display changes in the VZV-specific CD4:CD8 ratio according to IFNγ or TNFα secretion.

6.6 Discussion

The aim of this project was to find out the reasons behind the high incidence of VZV new infection and reactivation in leukaemic children under chemotherapy. Since the prevention of new infection and/or control of latent virus needs integration of different arms of the immune system, loss of VZV-specific B cell humoral immunity and/or T cell mediated immunity was hypothesised as a reason for this susceptibility. Furthermore, identification of an immune risk profile that can help in predicting leukaemic children who might be at high risk of VZV would be of great benefit. Therefore, anti-VZV IgG antibody titre (as indicator of B cell immunity) and VZV-specific T cell absolute count/ frequency and cytokine profile were monitored throughout this study.

In the current project more focus was given to T cell immunity including frequency, absolute count, and ratio of VZV-specific CD4+ and CD8+ T cell as well as their cytokine profile. This was based on several clinical observations that proposed T cells are more important than humoral immunity in recovery from VZV acute infection (Arvin, 2000, Schmader, 2001, Baiker *et al.*, 2010). Furthermore, the importance of helper CD4 T cells in activation of B cell and antibody class switching is well established (Parker, 1993).

In most previous studies, the determination of VZV-specific T cell mediated immunity was usually carried out using proliferation assays (Giller *et al.*, 1986, Haining *et al.*, 2005), which need several days of *in-vitro* PBMC incubation with VZV antigen. In the current study, applying such assays was impractical as cell viability, which is very crucial for such kind of assay, might be severely compromised by the cytotoxic chemotherapy. Therefore, an intracellular cytokine staining assay (ICS assay), which is

quick ex-vivo with short-term incubation periods, was optimized for present project. In addition, several strategies were introduced to improve the cell viability, for instance to avoid any toxic effect of drugs blood sampling was scheduled to be taken just before the chemotherapy cycle and samples were processed on the day of collection.

The T cell response in healthy control adults to VZV lysate from two different companies (East Coast Biologics and ABI companies) and two live attenuated VZV vaccines (Varivax and Varilrix) were tested in the current study. Both VZV-specific CD4+ and to a lesser extent CD8+ T cell responses were detectable by both ICS and proliferation assays. Nevertheless, implementation of proliferation assays in leukaemic children was confounded by both low cell viability and high background proliferation of nonspecific cells. N.b. in the ICS assay the T cell response was considered positive if the cytokine production of T cell stimulated by VZV antigen exceeded that of the mock response by 77% for CD4+ T cells and 30% for CD8+ T cells.

The current study confirms that VZV-T cell responses in adult healthy controls and leukaemic children were characterised by a predominance of CD4+ T cells which represent almost two thirds of the T cell response compared to CD8+ T cells which only comprise less than one third. This was somewhat unexpected as viral clearance relies heavily on CD8+ T cell functions, however, an increasing body of evidence has highlighted the importance of CD4+ T cells in directly regulating antiviral responses through proinflammatory cytokines acting in both a direct and indirect manner (Davis *et al.*, 2008). In accordance with these results, previous research demonstrated a similar observation (Arvin, 1996, Abendroth *et al.*, 2000, Jones *et al.*, 2006, Haberthur *et al.*, 2011). In addition, this might be explained by the high ability of VZV to evade CD8+ T

cell recognition through down regulation of MHC class I compared to the ability to escape CD4+ T cell recognition (Cohen, 1998, Abendroth and Arvin, 2001, Abendroth *et al.*, 2010). In addition, there might be an underestimation of CD8+ T cells because VZV antigen used in the majority of studies (including present study) is poorly presented through MHC class I (Abendroth and Arvin, 2001). Several VZV antigens have been identified as targets of CD4+ T cell compared to the partial understanding of VZV-specific CD8+ T cell specificity. For instance, CD4+ T cells were found to be able to recognize immediate early antigens, such as IE62, IE63, ORF4, and ORF10, and late antigens that included gB, gC, gI, gH, and gI surface glycoproteins (Arvin *et al.*, 1986, Bergen *et al.*, 1991, Arvin *et al.*, 2002).

One of the interesting observations of the present study was the change in the total CD4:CD8 T cell ratio in leukaemic children, which might be explained by unbalanced CD4+ and CD8+ population reduction that probably has more influence on CD4+ than CD8+ T cells. Such inversion in the ratio might have its importance as an immune risk indicator. This is supported by the fact that inversion in the CD4:CD8 T cell ratio is associated with morbidity and mortality in the elderly and considered as an immunosenescence indicator (Huppert *et al.*, 2003, Wikby *et al.*, 2005, Cretel *et al.*, 2011).

Having seen such changes in the total CD4:CD8 ratio, the anticipation was to discover a similar change in the ratio of VZV-specific T cells. Surprisingly, opposite to expectation, the frequency of VZV-specific CD4+ and CD8+ T cells in leukaemic children was very similar to that seen in the healthy adult controls. Furthermore, there was no significant difference in the CD4:CD8 ratio (VZV-specific) between both groups. This finding

might suggest that leukaemia affects mainly naïve and effector cells while the pool of memory cells, including VZV-specific memory T cells, is preserved. In support of this suggestion is the work by Haining and colleagues (Haining *et al.*, 2005) who showed that the antigen-specific T-cell memory is preserved in children treated for acute lymphoblastic leukemia.

Considering the relative stability of the VZV IgG titre during leukaemia and having seen no difference between healthy controls and leukaemic children regarding the frequency of VZV-specific T cells, the question here is, why leukaemic children are not protected against VZV, in other words what is the reasons behind the frequent reactivations of latent VZV. To answer this question, the cytokine profile of VZV-specific T cells was investigated. Taking in consideration that the T cell response to VZV has a T_H1 profile with little or no production of T_H2 cytokines (Watson *et al.*, 1995, Abendroth *et al.*, 2000, Vossen *et al.*, 2004); the two cytokines that were selected for measuring in this project were IFNγ and TNFα. Surprisingly, the current study demonstrates a significant reduction in the absolute numbers of VZV-specific IFNγ+CD4+ T cells, which seemed to be replaced by an expansion of TNFα+CD4+ T cells in leukaemic children. This finding might be of particular importance as there is evidence that supports a clear link between the early production of IFNγ but not TNFα (nor other Th1 cytokines) and the nature of the cellular immune response, and disease outcome in a virus model (Chaudhri *et al.*, 2004). Indeed this finding raises the possibility that inappropriate cytokine responses may be the cause of increased susceptibility to VZV infection in leukaemic children. However, since not all leukaemic children experienced VZV infection during the chemotherapy course, then it was reasonable to ask if there was any cytokine profile

that might be associated with VZV clinical infection. Therefore the patient group was sub-classified into three groups including a sub-group that had clinical VZV infection (group C) and groups with no clinical VZV infection (groups A and B), see Table 6-6 for detailed description of these groups.

Interestingly, the current study showed remarkably low absolute counts of VZV-specific IFNγ+ CD4+ T cells in group C. Furthermore, there was inversion of the ratio of VZV-specific CD4:CD8+ T cells that produce IFNγ. Based upon that, it seems conceivable to propose that both the reduction in the absolute counts of VZV-specific IFNγ+ CD4 T cell and/ or inversion of the VZV-specific CD4:CD8 (IFNγ producer) T cell ratio are an immune risk profile for VZV infection in leukaemic children.

However, it was not clear, whether these changes in immune profile preceded clinical VZV infection by a sufficient period and thus could be used as predictors for VZV reactivation, or that they took place just around the time of clinical appearance of VZV and therefore it were not appropriate for this purpose. Since the VZV incubation period can be 1 to 3 weeks before appearance of clinical manifestation (Arvin, 2000), that might mean actual viral replication might start a few weeks before clinical diagnosis. Therefore, it can be speculated that the changes in the immune system, particularly T cell immunity, may also precede the clinical manifestation. Nevertheless, according to the literature the T cell response is only detectable after the start of the clinical symptoms. However, most of these studies used proliferation assays to measure the VZV cell-mediated immunity with insufficient data on the ICS assay that is employed in this project. However, in the current study, prospective follow up of a couple patients who were VZV seropositive and had clinical VZV infection (most likely reactivation),

confirmed the proposal that the second infection was typically preceded by a drop in the VZV-specific IFNγ+ CD4 T cell absolute count with inversion in the CD4:CD8 ratio of VZV-specific T cells that produced IFNγ.

In contrast, the level of anti-VZV IgG antibody titre was high even before reactivation of VZV and it showed no correlation with T cell immunity. This might suggest that VZV-specific cellular immunity is more important than humoral immunity in preventing VZV reactivation. This notion was supported by clinical observations, for instance, children suffering from acquired immune deficiency syndrome (AIDS) have a significantly higher risk of progressive VZV reactivation (Gershon *et al.*, 1997, Adityan *et al.*, 2009), in contrast, children with agammaglobulinaemia usually have no increase in the risk of VZV infection (Webster *et al.*, 1988). Therefore, it might be inappropriate to rely only on VZV-specific IgG antibody titre as an indicator for protection against VZV. Furthermore, since the changes in antibody titre do not immediately reflect those in B cells, in other words it takes a while for antibody levels to drop even in the absence of the secreting B memory cells, this might be another reason for not considering VZV-specific IgG titre as an indicator of B cell protection in the context of leukaemia.

Therefore, implementation of other technique such as Fluorescent Antibody To Membrane Antigen assay (FAMA) and Time-Resolved Fluoro Immune Assay (TRFIA) assay to determining the VZV-specific B cell immunity (Dickson *et al.*, 1995), are being optimized by our collaborator Professor Judy Breuer and her colleagues at University College, London. Furthermore, incorporating VZV-specific ELISPOTs in the current

study is being considered as well.

As discussed in previous chapters, the control of CMV carriage is associated with marked changes in the immune system. This heavy investment in the response against a single virus may compromise T cell repertoire diversity and the ability to respond to other pathogens. Therefore, it can be hypothesised that carriage of CMV can be detrimental also to the immunocompromised host by suppressing heterologous VZV-specific immunity. This notion can be supported by earlier observations that showed CMV infection could reduce prevailing levels of immunity to another persistent virus such as Epstein-Barr virus (EBV) (Khan *et al.*, 2004). Furthermore, in agreement with that, in solid organ recipients, CMV replication was found to increase the viral load of other viruses, such as hepatitis C virus (HCV) (Razonable *et al.*, 2002). However, in context of ALL in children, this might be debatable as the incidence of CMV infection in this age group is relatively low in developed countries. Furthermore, the influences of CMV on the immune system seem to be related to duration of CMV carriage, in other words it is age related with older subjects being more severely affected. Nonetheless, investigating the impacts of simultaneous CMV infection upon immunity to other pathogens in ALL children is highly recommended. Moreover, another important question that might need to be addressed here is; what is the effect of VZV status on CMV immunity? This question is based on the conceivable role of HHV-6 and HHV-7 as copathogens in the direct and indirect illnesses caused by CMV(Razonable and Paya, 2002).

7 General Conclusion

This thesis set out with the aim of understanding the balance between the immune system and two common herpesviruses chronic infection, namely CMV and VZV. It was designed initially to explore the influence of chronic cytomegalovirus infection on the immune system of healthy subjects, particularly T cell immunity. Then, the impact of immune modulation by this virus on the sequelae of coincident coronary artery disease was investigated.

Meanwhile, the purpose of the VZV study was to determine the importance of adaptive immunity (B or T cell mediated immunity) in protection against VZV reactivation in children with acute lymphocytic leukaemia receiving chemotherapy. Besides, it evaluated whether the loss of VZV-specific B and/or T cell mediated immunity might be utilized as a risk predictor for VZV reactivation in patients who were considered to be protected.

To achieve these aims, four different projects were run simultaneously. However, since each project was independent of other, the results of each project were discussed in detail separately in its related chapter. Therefore, the purpose of this part of the thesis is to draw general conclusion from these four projects.

7.1 CMV work

7.1.1 Imprint of chronic CMV on the immune system of healthy subjects.

Initially, a pilot study was carried out aiming to explore simultaneously influences of CMV chronic infection on different lymphocyte subsets. Contrasting with most previous

studies this study considered these influences in the same subjects. The most obvious finding emerging from this study is that, compared to CMV-seronegative donors, the CMV-seropositive cohort exhibited a remarkable increase in the frequencies of $CD28^{neg}$ CD4+ (**Error! Reference source not found.**), $CD28^{neg}$ CD8+ T cells (**Error! ference source not found.**), $NKG2C^{pos}$ NK Cells (Figure 3.10) and $V\delta2^{neg}$ γδ T cells (**Error! Reference source not found.**). In addition, this study confirms, to some extent, hat these concurrent changes in phenotype are probably restricted to CMV infection and are not general phenomena that might occur with any chronic latent viral infection (see section 3.4.3). Furthermore inter-donor association analysis showed no statistically significant correlation between these subsets, which suggests independency of these cell subsets in controlling of CMV chronic infection, see section 3.4.5.

Indeed, participation of all of these subsets of lymphocytes in keeping CMV under control raises awareness of the major burden on the immune system of this virus. Furthermore, it might imply that development of a successful vaccine against CMV should consider activating all these subsets. Finally, in the current thesis results of this pilot study served as a basis for further investigations in chapters four and five.

7.1.2 Association between high frequency of $V\delta2^{neg}$ γδ T cells and CMV is age related

Since the importance of γδ T cells, principally, $V\delta2^{neg}$ subsets, in CMV infection has been studied only recently, and due to the real gap in knowledge about these cells, more investigation of this lymphocyte subset was carried out in this thesis. Based on the fact that the persistent nature of CMV infection is thought to drive the accumulation of large CD4+/CD8+ T cell expansions over time, the target of the current work was to test the

hypothesis that Vδ2^{neg} γδ T cell expansions occur more frequently in CMV carriers of older age and also to examine whether there are similarities between the phenotype and properties of Vδ2^{neg} γδ T cells and that of αβ T cells. The present study has shown increased levels of Vδ2^{neg} γδ T cells with advance in age in CMV-seropositive compared to CMV-seronegative donors.

Furthermore, it has demonstrated the Vδ2^{neg} γδ T cell phenotype is very similar to that of CMV-specific CD4+ and CD8+ T cells. In particular, Vδ2^{neg} γδ T cells were akin to CMV-specific CD8+ T cells; both are mainly T_{EM} cells, including CD45RA^{high} T_{EMRA} cells, with a highly differentiated CD27^{low}CD28^{low} phenotype in Vδ2^{neg} γδ T cells (see section 4.5.8). Moreover, Vδ2^{neg} γδ T cells were found to express high levels of perforin and granzyme B, indicating cytotoxic functionality of this T cell subset.

In addition, the reactivity of Vδ2^{neg} γδ T cells against CMV-infected target cells was confirmed using in vitro expanded γδ T cell lines. Taken together, this state of readiness provides support for these cells having a central role in the immune response early in primary infection. However, it was not possible to demonstrate immediate effector activity in *ex vivo* assays, which was unexpected given the shared effector memory phenotype of Vδ2^{neg} γδ T cells and virus-specific αβ T cells. Taking into consideration that the majority of Vδ2^{neg} γδ T cells are located in peripheral tissues, these results suggest that, Vδ2^{neg} γδ T cells can be triggered directly by infected cells, while viral reactivation from latency or cross-presentation by APCs can lead to efficient stimulation.

7.1.3 CMV in coronary artery disease (CAD)

The purpose of this part of the project was to investigate the possible role of subclinical reactivation of latent CMV in coronary artery disease. Therefore differences in the magnitude of T cell responses to CMV antigens in CAD and age matched healthy controls were tested by flow cytometry. While a variety of definitions of the term "quality of T cell responses" have been suggested, in this chapter of the thesis, it refers to both the frequency as well as the pattern of T cell responses against CMV antigens. The "pattern of function" is related to how many functions the CMV–specific T cells can perform simultaneously, which ranges from mono to multifunctional (Betts *et al.*, 2006, Darrah *et al.*, 2007, Seder *et al.*, 2008). Therefore, the description of T cell quality included three aspects: effector function, proliferation ability, and cytotoxic capability. However, since there is no consensus about which markers should be used for these purposes, the justification for selecting the panel of markers that was used in this project is discussed in the following paragraphs.

Undoubtedly IFNγ should be included in the panel as correlation between the frequency of IFNγ producing cells and clearance of various pathogens, particularly viruses, is well established (Ouyang *et al.*, 2003b, Khan *et al.*, 2007). Nevertheless, several observations showed that the frequencies of IFNγ producing cells do not adequately reflect the level of protection (Elias *et al.*, 2005, Duvall *et al.*, 2008). The ability of TNFα to mediate the killing of infected cells independently of IFNγ makes it second in the list of cytokines that are proposed to be measured (Herbein and O'Brien, 2000, Chan *et al.*, 2003, Simon *et al.*, 2005).

The third cytokine in the panel was IL-2, which has a major role in promoting the proliferation of CD4+ T cells and CD8+ T cells (Williams *et al.*, 2006, Sester *et al.*, 2008). In addition, it enhances the activation of NK cells, which contribute to infection eradication.

Regarding cytotoxicity, there are two ways to measure the capability of T cells to kill, particularly CD8+ T cells. These are either by directly measuring intracellular cytotoxic granules contents such as perforin and granzymes or indirectly through measuring a degranulation marker, CD107a/b (Betts *et al.*, 2003). Again due to limitation in the number of flow cytometer channels it was decided to use the CD107a immobilization technique to measure the cytotoxic activity. To make coverage of all aspects of T cell response quality more complete, a chemokine was added to the proposed panel. MIP1β (CCL4) was selected, in view of the possible role of this chemokine in immunity against CMV. For instance, MIP1β was found to be capable of inhibiting HIV-1 infection by binding with the HIV-1 co-receptor CCR5 (Cocchi *et al.*, 1995). Recently, CMV latent memory T cells were found to have a distinct cytokine signature characterized by high MIP1β expression (Kim *et al.*, 2009a). In addition it plays important role in linking innate and adaptive immunity through its potent chemo-attracting function for monocytes and NK cells. Therefore, measurement of the components CD107a, MIP1β, IFNγ, TNFα, and IL-2 together can give a more comprehensive picture of the quality of the antigen-specific T cell response.

The most striking observation of this study was the monofunctional restriction of most responding CMV specific T cells in coronary artery patients. Interestingly, no one

chemokine/cytokine was consistently expressed in all CMV reactive donors, suggesting that measuring one cytokine might be not sufficient to assess the magnitude of the T cell response. Additionally, and in contrast to expectation, neither IFNγ nor TNFα were the most frequent cytokines produced by stimulated T cells, instead MIP1β was the predominant cytokine in term of frequency (see section5.4.8). This might be of particular importance in assays that rely on measuring IFNγ alone such as ELISPOT assays, which may seriously underestimate the actual frequency of antigen-specific T cells. Hence, it can be proposed that MIP1β is a more sensitive marker than IFNγ and TNFα for specific T cell activation.

With in the CAD group the most obvious differences between AMI (acute myocardial infarction) and PMI (stable post myocardial infarction) patients were in the distributions of polyfunctional CMV specific CD4+ T cells that were characterized by the absence of IFNγ but expression of IL-2 plus any other function. This cell subset was significantly higher in the PMI group, see section 5.4.11. This might suggest the importance of IL-2 as a stability factor in coronary artery disease in CMV carriers. An implication of this is the possibility that the presence of (IFNγ- IL-2+) CMV specific CD4+ T cells could be used as an immune biomarker in CAD patients. In other words, absence of this functional pattern could be used to identify CMV-seropositive CAD patients at risk of developing AMI and hence to help development of novel strategies for future treatment and prevention.

7.1.4 Should CMV be eradicated?

Having seen all of these significant alterations in the immune system of CMV carriers, this could lead to the general feeling that CMV is bad for our health! Thus, preventing

infection by vaccination or even eliminating the established infection with antiviral drug such as ganciclovir may be desirable. This idea was augmented by proposing a link between CMV chronic carriage and high incidence of many infectious diseases in the elderly. One mechanism that sheds light on the association between CMV and morbidity in the elderly is the remarkable shrinkage in the available naïve T cell repertoire. This is attributed to the consequence of competition for 'immunological space' by dysfunctional CMV-specific T cell clones and other novel antigen-specific cells (Ouyang *et al.*, 2004). However, It might be debated that expanded CMV-specific T cell clones have some beneficial effects, for instance, severe CMV infection is rarely reported in the immune-competent (Effros, 2005). Furthermore, the presence of high frequencies of highly differentiated ($CD28^{low}$) CD8+ T cells post transplant has been correlated to low risk of acute graft rejection (Colovai *et al.*, 2003, Nickel *et al.*, 2009) which might suggest a suppressive effect of these cell subsets (Liu *et al.*, 1998). Nevertheless, this suppressive role can be responsible for failure of influenza vaccination in the elderly (Effros *et al.*, 2005, Song *et al.*, 2010).

Therefore, as a personal opinion, eradication of chronic CMV infection might be a reasonable goal only in certain circumstances when virus activation can be devastating. An example of that is post bone marrow transplant. There is, therefore, a definite need for a novel CMV vaccine. However a successful vaccine has to be able to mimic the response to the natural infection (Lambert *et al.*, 2005), which in CMV incorporates all arms of the immune system. Taking into consideration that the burden of natural CMV infection is related to its influences on the immune system rather than to its direct tissue damage, then it is reasonable to argue against this vaccine. Furthermore, to date, there is

no approved CMV vaccine though there is an encouraging report that CMV vaccination can prevent CMV primary infection in pregnant seronegative mothers (Pass *et al.*, 2009). The ambition for a novel CMV vaccine that can eradicate CMV infection from society may be achievable one day. However, the impact of that will remain an open question for more debate.

7.1.5 Novelty of CMV work

The influences of CMV infection on different arms of the immune system have been extensively studied, this was reviewed elsewhere (Khan, 2007). This includes the influences on the lymphocyte subsets that are studied in this thesis, namely highly differentiated ($CD28^{low}$) αβ T cells, $NKG2C^{pos}$ NK cell, and $V\delta2^{neg}$ γδ T cells. However, the originality of this thesis is related to the exploring of changes in these lymphocyte subsets in the same subject. And according to the best of our knowledge it is the first study that addresses the correlation between; conventional (αβ), non-conventional (γδ) T cells (both are adaptive immune system) and NK cells (innate immune system).

Although the CMV seropositivity and the expansion of $V\delta2^{neg}$ γδ T cells has been established in both immunocompromised subjects (Dechanet *et al.*, 1999a, Knight *et al.*, 2010) and immunocompetent subjects (Pitard *et al.*, 2008), the current study is the first detailed account of how γδ T cell subsets are skewed by the combined effects of aging and CMV carriage in healthy individuals.

In the context of influence of CMV status on the immune system of the diseased, the current study showed for the first time that there is a difference in the CMV-specific T cell profile of AMI and PMI patients. Therefore, this study has gone some way towards

suggesting the influence of CMV chronic infection on the immune system as an aetiological factor in ischemic heart diseases rather than CMV as direct causative factor.

7.1.6 Limitations of current CMV work

These conclusions should be considered in the light of some limitations. For example, the initial pilot study (chapter 3) was limited by the ability of the flow cytometer to detect only four colours, preventing comprehensive phenotyping and sub-classification of lymphocytes. Another limitation was the age of the cohort, which was between 20-60 years, while it is known that CMV has more prominent effects in the age group over 60 years. In addition, with a small sample size of primary acute CMV infection work (only two cases were accessible), caution must be applied, as findings might not be transferable to larger populations.

While an 8-colour flow cytometer was available for the CAD project (chapter 5), the use of frozen samples for functional assays might be a drawback. In addition, the lack of phenotypic markers to differentiate memory from effector T cell subsets is another limitation.

7.1.7 Future work on CMV

No doubt formal identification of the ligand(s) for the TCR of γδ T cells would help in answering many important questions of immense value for vaccine design, a major priority for CMV (Arvin *et al.*, 2004), where the aim should be to elicit broad multi-specific immunity (Khanna and Diamond, 2006). It will also be important to learn if

CMV targets γδ T cells for immune subversion, as is the case for other T cells and for NK cells (Powers *et al.*, 2008).

Recently, Vδ2^{neg} γδ T cells from CMV+ kidney transplant patients were found to express the IgG Fc receptor CD16 and to show enhanced function in the presence of anti-CMV antibody (Couzi *et al.*, 2012). This emphasizes the potential for collaboration between Vδ2^{neg} γδ T cells and IgG in mediating antibody-dependent cellular cytotoxicity. It would be of interest to study CD16 expression by Vδ2^{neg} γδ T cells in leukaemia patients with or without VZV reactivation in relation to antibody titres.

Given the shared reactivity of Vδ2^{neg} γδ T cells for CMV and epithelial tumour cells (Halary *et al.*, 2005) and the greater incidence of cancer in the elderly, this finding has great significance. Indeed, CMV seropositivity and increased numbers of Vδ2^{neg} γδ T cells coincide with lower risk of cancer in a long-term follow up of kidney transplant patients (Couzi *et al.*, 2010). Further independent studies of CMV status and Vδ2^{neg} γδ T cells in cancer patients are thus a major priority for the future.

Studying the role of CMV in CAD has thrown up many questions in need of further investigation. For instance, additional experimental investigations are needed to establish whether CMV viral load correlates with polyfunctionality and T cell quality during acute myocardial infarction. Indeed, correlating changes in telomere length to CMV polyfunctionality is another potential area for research. In addition, a longitudinal cohort study to monitor the dynamic changes in cytokine profile during and after acute myocardial infarction would be of great help in understanding the role of CMV infection

in coronary artery diseases. Furthermore, supplementary information on the role of other common latent viral infection such as herpes zoster is also needed.

7.2 VZV work

The main aim of the VZV study was to find an explanation for the high incidence of VZV reactivation in leukaemic children. Furthermore, as a secondary aim, it was tried to establish an immune risk profile that could help in identification of leukaemic children who might be at risk of VZV reactivation. The change in both VZV-specific B cell responses (through measuring of VZV-specific IgG titre by ELISA) and frequency and cytokine profile of VZV-specific T cell were monitored in this prospective study.

One of the more significant finding to emerge from this study is the significant reduction in the absolute count of VZV-specific IFNγ+ CD4+ T cells in those leukaemic children group who developed clinical VZV infection (see section 6.5.13). This reduction was associated with an inversion in the CD4:CD8 ratio of VZV-specific T cell populations that produce IFNγ. It was possible to detect this profile during acute infection and even a few weeks before appearance of infection in a previously naturally immunized patient (see section 6.5.15). This may lead to the proposal that both the reduction in the absolute counts of VZV-specific IFNγ+ CD4 T cells and/ or inversion of the VZV-specific

CD4:CD8 (IFNγ producer) T cell ratio are an immune risk profile for VZV reactivation in leukaemic children.

Contrasting with T cell responses, VZV-specific IgG was detected post primary infection in most patients; however, there was no clear association between the VZV-specific IgG antibody titre and VZV reactivation. In general, therefore, it seems that VZV-specific IgG antibody titre might be an inappropriate indicator for protection against VZV reactivation in the context of leukaemia.

7.2.1 Novelty of VZV work

The VZV work was original theoretically and methodologically. For instance, to the best of our knowledge, the current VZV study is the first prospective longitudinal study that has explored B cells and/or T cells as a possible immunological defect behind the high incidence of VZV reactivation in children with ALL. Methodologically, the ICS assay was modified by using whole attenuated viral vaccine as stimulus instead of using peptides mix; also the assay was optimized to work with limited numbers of cells in immune compromised patients.

7.2.2 Limitations of current VZV study

A number of important limitations need to be considered in VZV project. First, it is worth noting that this clinical study is still running, and since only few patients have completed their chemotherapy course, the data presented in this thesis can be considered as preliminary. In addition, it is admitted that the absence of age matched healthy controls is one of the weaknesses of this project. Therefore, recruiting healthy children from those children who were admitted for elective surgery such as tonsillectomy might

be an option. Furthermore, the current study was limited by the gap in knowledge about other lymphocytes such as NK cells and unconventional T cell subsets.

7.2.3 Future work on VZV

What is now needed is continuous recruitment of more patients and monitoring them for a longer period. Furthermore, the use of ultra sensitive and highly specific assays such as TRFIA, FAM and ELISPOT would be of great importance in assessing B cell immunity (O'Ferrall, 1954, Baba and Shigeta, 1986). In addition, further investigation and experimentation into the role of innate immunity, particularly of NK cells is strongly recommended. It has been shown that the NK cell KIR B haplotype, which contains several activating receptors, is associated with increased protection against CMV reactivation after bone marrow stem cell transplantation (Cook et al, 2006). It would be of interest to examine KIR genotypes in leukaemia patients who do or do not subsequently become infected with VZV.

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!!! INVALID CITATION !!!

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